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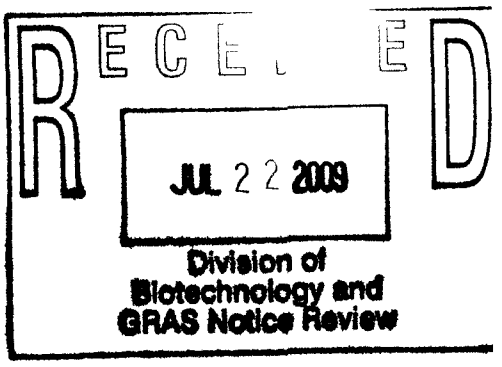
ORIGINAL SUBMISSION

JHeimbach LLC



July 16, 2009

Robert L. Martin, Ph.D.
Deputy Director
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Bob:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), BioVittoria Ltd., of Hamilton, New Zealand, through me as its agent, herby provides notice of a claim that the use of Luo Han Fruit Concentratie in conventional foods as described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because BioVittoria Ltd. has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, three copies of the notification are provided. Additionally, three copies are provided of the Conclusion of the Expert Panel, including the signatures of the three members of the panel, in Appendix III of the notification.

If you have any questions regarding this notification, please feel free to contact me at 804-742-5548 or jh@jheimbach.com.

Sincerely,

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

923 Water Street, P.O. Box 66, Port Royal Virginia 22535, USA
tel. (+1) 804-742-5548 fax (+1) 202-478-0986 jh@jheimbach.com

GRAS Exemption Claim for the Use of Luo Han Fruit Concentrate as a Flavor Modifier and Sweetener in Conventional Foods

1. Name and Address of Notifier

BioVittoria Ltd.
Waikato Innovation Park
Ruakura Road
P.O. Box 9466
Hamilton, New Zealand

Contact: David Thorold, Chief Executive Officer
Telephone: 64 7 857 0521
Facsimile: 64 7 857 0501
E-mail: david@viovittoria.com

2. Name of GRAS Substance

The subject of this Generally Recognized as Safe (GRAS) determination is a clarified concentrate derived from Luo Han Guo (*Siraitia grosvenorii* Swingle) trademarked and sold as PureLo® brand Luo Han fruit concentrate. The primary components of the fruit, responsible for its sweetness, are cucurbitane glycosides known as mogrosides.

3. Intended Use and Consumer Exposure

Luo Han fruit concentrate is intended to be added to conventional foods at the concentration needed, consistent with cGMP, as a flavor modifier and sweetener. It may also be used as a tabletop sweetener. It may be used alone or as a component in sweetener blends. An extremely conservative estimate of the potential 90th percentile intake of the substance from its intended use is 6.8 mg/kg bw/day, a level that could be reached only if this substance were to capture the entire market for intense sweeteners.

4. Basis for GRAS Determination

BioVittoria's GRAS determination for the intended use of Luo Han fruit concentrate is based on scientific procedures as described under 21 CFR §170.30(b). Determination of the safety and GRAS status of the intended use of Luo Han fruit concentrate was made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D,, and John A. Thomas, Ph.D., who reviewed a monograph prepared by JHeimbach LLC as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They critically reviewed and evaluated the publicly available information and the potential human exposure to Luo Han fruit concentrate resulting from its intended use and individually and collectively concluded that no evidence exists in the available information that demonstrates or suggests reasonable grounds to suspect, a hazard to adults or children under the intended conditions of use of Luo Han fruit concentrate.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, the intended use of Luo Han fruit concentrate is GRAS by scientific procedures.

5. Availability of Information

The data and information that serve as the basis for this GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of James T. Heimbach, Ph.D., President, JHeimbach LLC, 923 Water Street, P.O. Box 66, Port Royal, Virginia 22535, telephone 804-742-5548 and e-mail jh@jheimbach.com.

Determination of the GRAS Status Of the Use Of Luo Han Fruit Concentrate As a Flavor Modifier and Sweetener

Prepared for
BioVittoria Limited
Hamilton, New Zealand

Prepared by
JHeimbach LLC
Port Royal Virginia

July 2009

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Determination of the GRAS Status Of the Use Of Luo Han Fruit Concentrate As a Flavor Modifier and Sweetener

1. IDENTITY OF THE SUBSTANCE

1.1. Name and Description of the Substance

The food that is the subject of this Generally Recognized as Safe (GRAS) determination is a clarified concentrate of a decoction of Luo Han Guo (*Siraitia grosvenorii* Swingle) trademarked and sold as PureLo® brand Luo Han fruit concentrate by BioVittoria Limited of New Zealand. Concentrations of such decoctions have been consumed as food and used as sweeteners in China for centuries and sales of the fruit have been documented in the U.S. since the late 1800s. The methods used in the production of PureLo® Luo Han fruit concentrate do not differ remarkably from those used to produce other fruit-derived products widely consumed in the U.S.

The basionym for Luo Han Guo is *Momordica grosvenorii* Swingle (Swingle 1941). The fruit was moved from the genus *Momordica* to *Thladiantha* in 1979 (Jeffrey 1979) and to *Siraitia* in 1980 (Jeffrey 1980, 1990a). Jeffrey (1990b) included all three genera and the genus *Indofevillea* in the same subtribe (Thladianthinae) of the tribe Joliffleae (which contains one additional genus, *Terfairia*), and the subfamily Cucurbitaceae. Common names include Luo Han Guo, Luo Han Kuo ("guo" or "kuo" is the Chinese for "fruit"), Lo Han Kuo, Lo Han Guo, Lor Hon Kor, Rah Kan Kah, Arhat Fruit, Fructus Momordicae, Longevity Fruit, Monk's Fruit, and (in Japan) Rakanka.

The original botanical description of *Siraitia grosvenorii* was published in 1941 by W.T. Swingle from plants collected in southern China (Swingle 1941). Swingle named the plant *Momordica grosvenorii* in honor of Dr. Gilbert Grosvenor, the president of the National Geographic Society, the sponsor of a 1937 expedition to collect Luo Han Guo in China by Professor G.W. Groff. (In light of its later taxonomic reclassification, it is interesting to note that, while Swingle regarded Luo Han as a new species of *Momordica*, he noted that it is "very distinct from any now known to botanists" [Swingle 1941].) In 1941, Swingle described the plant as a cultivated dioecious vine with bifid tendrils, climbing 2-5 meters, with tuberous, perennial roots. The fruits and leaves of the four principal cultivated varieties were described as showing "rather striking differences in the shape and color of the fruit and in the shape and size of the leaves..." Fruits of plants that were of wild origin were not studied by Swingle (Swingle, 1941).

As noted, Luo Han is a member of the genus *Siraitia*, which contains only three other widely separated species: *S. siamensis* (Thailand), *S. silomaradjae* (India), and *S. taiwaniana* (Taiwan). However, it is also a member of the subfamily Cucurbitaceae, which includes the cucumber, melon, watermelon, squash, gourd, and other commercially important species. This subfamily is distinct morphologically and biochemically from

other families and is therefore considered monophyletic.

1.2. Chemical Names

PureLo® is a fruit concentrate, in either liquid or powder (spray-dried) form, comprising a mixture of naturally occurring compounds found in the fruit of the Luo Han plant. Thus there is no single chemical name for the food. The primary components are cucurbitane glycosides known as mogrosides, specifically mogrosides II, III, IV, V, and VI, along with flavonoids and melanoidins (formed from degradation of ascorbic acid and lipids, and as Maillard reaction products during processing). Mogroside V is the major component, constituting over 30% of the product, and is primarily responsible for the sweetness of Luo Han fruit decoctions.

Mogroside V is also known as mogro-3-O-[β-D-glucopyranosyl (1-6)-β-D-glucopyranoside]-24-O-{[β-D-glucopyranosyl (1-2)]-[β-D-glucopyranosyl (1-6)]-β-D-glucopyranoside}. The systematic name of mogroside V is β-D-glucopyranoside, (3β,9β,10α,11α,24R)-3-[(6-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-11,25-dihydroxy-9-methyl-19-norlanost-5-en-24-yl-O-β-D-glucopyranosyl-(1-2)-O-[β-D-glucopyranosyl-(1-6)]-

1.3. CAS Registry Number

Because Luo Han fruit concentrate is a mixture of naturally occurring compounds, there is no Chemical Abstracts Service (CAS) Registry Number for the food product. CAS numbers do exist for three mogrosides: mogroside V, the principal component, is CAS #88901-36-4. The other two mogrosides with CAS numbers are mogroside IV (CAS #89590-95-4) and mogroside VI (CAS #89590-98-7).

1.4. Molecular and Structural Formulas

The mogrosides are highly stable molecules based on a cucurbitane skeleton. As the name suggests, the cucurbitacins occur predominantly in the family Cucurbitaceae, and can be found in many groups of plants in the cucumber family, of which Luo Han is a member. Cucurbitacins are a group of secondary plant metabolites, classified chemically as triterpenes based on the 19-(10→9β)-abeo-10α-lanost-5-ene (cucurbitane) skeleton. All terpenoids are derived from repetitive fusion of branched 5-carbon isoprene units; the triterpenoids, which contain 30 carbon atoms, are generated by the head-to-head joining of two C_{15} chains, each of which contains 3 isoprene units joined head to tail.

Mogrosides are formed of varying numbers of glucose units, from 2 to 6, attached to carbon 3 and carbon 24 (indicated as R_1 and R_2 in Figure 1) on the triterpene backbone.

All of the mogrosides are classified as triterpene glucosides, designated as the diglucoside, triglucoside, tetraglucoside, pentaglucoside and hexaglucoside. Mogrosides IV, V, and VI are very sweet and are responsible for the sweetness of Luo Han fruit and consequently that of the PureLo® concentrate of Luo Han fruit. Mogroside V is the major sweetness component of the fruit, comprising up to 0.5% of the dried fruit weight. The

inherent robust stability of the coordinate covalent bonds between the triterpene framework and carbohydrate residues attached at carbons 3 and 24 render the mogrosides inert to thermal and enzymatic degradation. Thus mogrosides are biochemically stable, non-nutritive, and non-hygroscopic[1].

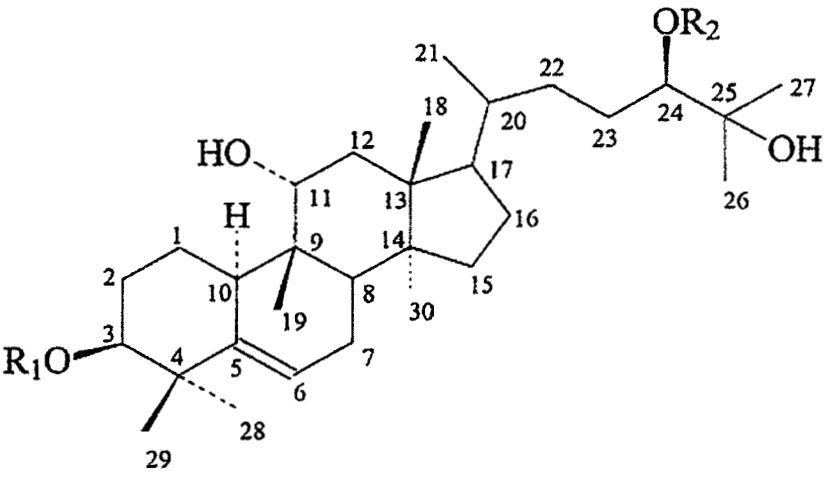

Figure 1. Molecular Structure of the Triterpene Backbone.

The simplest mogroside, mogroside II, has one glucose residue attached to each of carbons 3 and 24. Mogroside III differs in having an additional glucose residue chained to carbon 24, while mogroside IV has 2-unit glucose side chains at both carbon 3 and 24. This progression continues through mogroside VI, which has 3 glucose residues attached at each of the two carbons at locations 3 and 24 of the triterpene backbone. This is illustrated below in Figure 2.

[1] In a study of the triterpene glycosides and polyphenols found in black cohosh, Jiang et al. (2008) found that, while the polyphenols were unstable at elevated temperature or humidity, the triterpene glycosides were stable in all tested conditions. Indeed, it was found that the triterpenoid content of an 85-year-old sample was similar to that of a fresh sample.

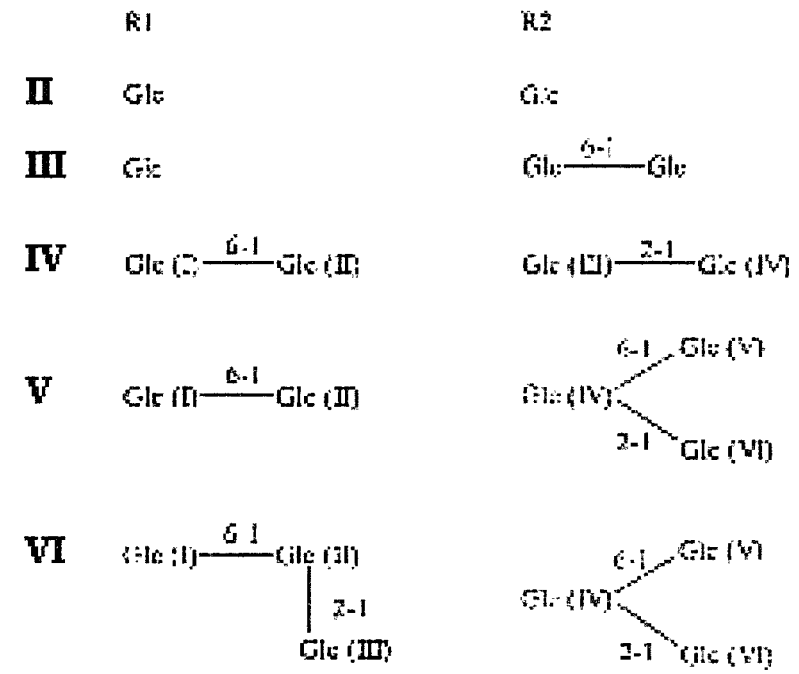

Figure 2. Mogroside II Through VI Side Chains.

The molecular formula of mogroside V is $C_{60}H_{102}O_{29}$; its molecular weight is 1286 Dalton (Da). The molecular formulas of the other mogrosides are as follows:

mogroside II:	$C_{42}H_{72}O_{14}$
mogroside III:	$C_{48}H_{82}O_{19}$
mogroside IV:	$C_{54}H_{92}O_{24}$
mogroside VI:	$C_{66}H_{112}O_{34}$

The structural formula of mogroside V is shown in Figure 3.

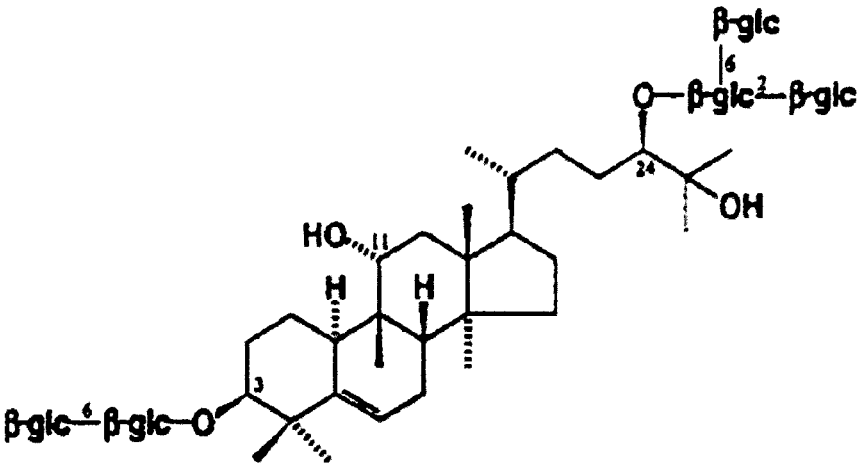

Figure 3. Structural Formula of Mogroside V.

1.5. Source Material and Production/Purification Process for PureLo

1.5.1. Proximate Composition of Luo Han Guo

The fruit comprises about 27% to 33% lipid in the form of triacylglycerols composed primarily of linoleic, oleic, and palmitic acids (People's Republic of China [PRC], Ministry of Agriculture, Center for Agri-food Quality and Safety 2009). The protein content averages about 26% with 18 amino acids identified but tryptophan lacking. The most prevalent amino acids are aspartic acid, serine, proline, and glutamic acid (PRC 2009). The Luo Han fruit is high in vitamin C, containing 340-488 mg/100 g fruit. The saccharide and polyol content of the fruit is about 2.4%, over 70% of it fructose and about 8% inositol (Hussain et al. 1990).

1.5.2. Mogroside Concentrations in Luo Han Guo

A number of investigators have studied the mogroside content of Luo Han fruit; all of these analyses have been based on the dried fruit, but they have employed different procedures as well as a variety of solvents, making comparisons across studies difficult or impossible. In 1985, Makapugay et al. found mogroside V contents of 0.80% and 1.29% by weight in two samples of dried Luo Han fruit. Concentrations were highest (1.37% and 1.56%, respectively) in the endocarp. Matsumoto et al. (1990) performed a methanol extraction from dried Luo Han fruit and identified 7 cucurbitane glycosides. These included the previously known mogroside V and mogroside IV, present at 0.45% and 0.034%, respectively. Four other mogrosides had been previously isolated in other species: 11-oxo-mogroside V (present at 0.18%), siamenoside I (0.044%), mogroside IIIE (0.029%), and mogroside IIE (0.025%). The seventh mogroside, which had never previously been isolated, was identified as mogroside III, present at 0.008%. 11-oxo-mogroside V differs from mogroside V only in having an oxygen instead of a hydroxyl group attached to carbon 11, while siamenoside I differs from mogroside II in having one glucose residue attached to carbon 3 and three glucose units to carbon 24 rather than two glucose residues attached to each carbon. In all of the identified glycosides, the glycosidic bonds are in β configuration.

Matsumoto et al. (1990) had expert tasters evaluate the sweetness of each isolated glycoside by adjusting the concentration of aqueous solutions until the sweetness seemed equivalent to a 5% sucrose solution. Mogrosides V and IV, as was already known, were very sweet, judged to be 425 and 392 times sweeter than sucrose, respectively. Siamenoside I also proved to be extremely sweet, judged to be 563 times sucrose, while 11-oxo-mogroside V was rated as 84 times sweeter than sucrose. The other three mogrosides were tasteless. Matsumoto et al. (1990) speculated that sweetness of a particular glycoside is dependent on both the number and allocation of glucose units.

Chang (1996) used spectrographic analysis to identify mogrosides II, III, IV, and V as well as a new glycoside that he named neomogroside, but which is systematically referred to as mogroside VI. The extraction method was not reported. Chen et al. (2005a), in an extensive review of cucurbitane glycosides, cited a study published only in Japanese in 1983 that reported isolating a mogroside with 7 attached glucose units, mogroside VII,

but this glycoside has not been reported elsewhere. The English abstract did not include specification of the extraction method.

Ukiya et al. (2002) performed a series of extractions from 2 kg dried and powdered Luo Han fruit to isolate and characterize 19 compounds. The solvents and their soluble compounds were:

- n-hexane soluble fraction:
  - 5-dehydrokarounidiol dibenzoate (2.7 mg)
  - karounidiol dibenzoate (11.4 mg)
  - triterpene monobenzoate (3.0 mg)
  - 3 triterpene mono-ols (12.0 mg)
- methanol soluble fraction:
  - mogrol (61 mg)
  - 5α,6α-epoxymogroside 1 E_1 (3.3 mg)
  - 11-oxo-mogroside I A_1 (31 mg)
  - 11-oxo-mogroside I E_1 (6.5 mg)
  - mogroside I A_1 (218 mg)
  - mogroside I E_1 (172 mg)
- butanol soluble fraction
  - mogroside II E (917 mg)
  - mogroside III (405 mg)
- water soluble fraction
  - siamenoside I (90 mg)
  - mogroside IV A (408 mg)
  - mogroside IV E (352 mg)
  - 11-oxo-mogroside V (366 mg)
  - mogroside V (2714 mg)

Clearly mogroside V is the principal glycosidic compound in Luo Han fruit, and this is even more so when only the water-soluble fraction is considered.

As part of a study to determine the potential of transglycosylation to improve the sweetness quality of mogroside V, Yoshikawa et al. (2005) evaluated the mogroside content of a Luo Han water extract containing about 36% (w/w) total mogroside. The contributions (w/w) of the individual mogrosides were: mogroside V = 84.2%, 11-oxo-mogroside V = 5.4%, mogroside IV = 4.4%, siamenoside I = 3.8%, and mogroside III = 2.2%. As noted previously, Yoshikawa et al. (2005) confirmed that the anomeric type of all glycosidic linkages is β-type.

A methanol extraction of unripe Luo Han fruits isolated two previously unreported triterpene glycosides, 20-hydroxy-11-oxo-mogroside I A_1 and 11-oxo-mogroside II E (Li et al. 2006). In follow-up work, again using methanol extraction from unripe Luo Han fruits, Li et al. (2007a) isolated three more glycosides, 11-oxo-mogroside III, 11-dehydroxymogroside III, and 11-oxo-mogroside IV A.

Using an extraction method in which air-dried and powdered Luo Han fruit was soaked in ethanol at room temperatures for 1 week, repeated three times, Akihisa et al. (2007) isolated 6 new triterpene glycosides. These were identified as mogroside II B, 11-

deoxymogroside III, 7-oxo-mogroside II E, 7-oxo-mogroside V, 11-oxomogroside II A_1, and 11-oxomogroside IV.

Xia et al. (2008) compared the efficiencies of three methods of extraction in maximizing concentrations of the principal sweet mogrosides, mogroside V, 11-oxo-mogroside V, and mogroside IV. The test methods were Soxhet (hexane:ethanol, 1:4 v/v), supercritical ethanol:carbon dioxide, and subcritical water. Subcritical water extraction (water under pressure at greater than 100°C) was found to be the best method.

The composition—especially the mogroside content—varies with the maturity of the fruit. Chen et al. (2005b) claimed to have found that the content of mogroside V increases rapidly after 50 days, stabilizing after about 80 days[1]. Li et al. (2007b) also studied the content of mogroside V, mogroside III, and mogroside II E at various stages of growth from 5-day-old fruit to 85-day-old fruit. As shown in Table 1, the concentration of mogroside V increased with age, stabilizing by day 80, while the concentrations of the other two tested mogrosides increased at first but then declined. Mogroside III reached its peak in 50-day-old fruit while mogroside II E content peaked at only 10 days; both of these mogrosides declined to non-detectible levels by 70 to 80 days.

Table 1. Content of Mogrosides V, III, and II E By Growth Stage.

Growth Stage of Fruit	Mogroside V (mg/g)	Mogroside III (mg/g)	Mogroside II E (mg/g)
5 days old	0.00	1.25	12.06
10 days old	0.00	2.61	28.21
20 days old	0.00	3.26	19.95
30 days old	0.00	4.04	12.40
40 days old	1.04	4.79	5.50
50 days old	5.50	6.06	1.17
60 days old	8.80	4.00	0.61
70 days old	10.50	1.15	0.00
80 days old	16.50	0.00	0.00
85 days old	16.30	0.00	0.00
Source: Li et al. (2007b); values are averages of 3 samples			

In summary, mogrosides appear to constitute about 0.5% to 1% of the weight of dried Luo Han fruit, with mogroside V making up well over half of this total in mature fruits. A large number of mogrosides or similar triterpene glycosides, which are variously soluble in methanol, ethanol, butanol, hexane, or water, appear to be present in the fruits at some stage of development. When the water-soluble fraction of Luo Han extracts is examined, mogroside V accounts for 69% of the mogroside content (Ukiya et al. 2002), while mogroside IV provides 19% and 11-oxo-mogroside V constitutes 9% of the total mogrosides. However, until recently no validated standards existed for water-soluble

[1] This claim was made in the abstract of the article, but none of the research reported in the article itself bears on the change in mogroside V content as fruit ripens.

mogrosides other than mogroside V (Lyndon 2006) and thus reported analytical findings must be interpreted with caution[1]. Since flavonoids and melanoidins absorb strongly around 600 nm, as do mogrosides, ultraviolet-visible (UV-Vis) spectroscopy may produce spuriously high estimates of total mogroside content.

1.5.3. Production of Luo Han Fruit by BioVittoria

The plants that are used to produce the Luo Han fruit which is to be processed into PureLo® Luo Han fruit concentrate are carefully selected and managed. Varieties for propagation are selected based on mogroside yield, resistance to drought and virus, and ease of cultivation. "Mother plants" from these varieties are used as the source of tissue for cultured Luo Han plants, which are protected by Plant Variety Rights owned by BioVittoria's Chinese subsidiary, Guilin Bio-GFS. Healthy bud tissue is selected, cleaned with deionized water, and then disinfected with sodium hypochlorite or silver chloride. The disinfected buds are placed on an agar medium to foster callus tissue and the development of differentiated buds, a stage of development that takes 40-50 days. The callused tissue is then transferred to a new agar medium that fosters the development of differentiated plants that have yet to grow roots, a stage requiring 200-240 days. The differentiated plants are moved to a medium that encourages the development of roots, where they spend 25 to 35 days, and then are transplanted to pots containing a potting mix specifically developed for the growth of Luo Han plants. The pots remain in a warm, high humidity shade house for 30-40 days. Finally, during March and April, the potted seedlings are transplanted to the fields of farmers who grow the Luo Han fruit under contract with BioVittoria.

The plants are grown using organic fertilizer enriched with phosphate and potassium during the early stages of growth. When necessary, natural pyrethreum is used to control insects, usually from the time of planting until mid-summer. The flowers first appear in late spring and continue until autumn; since male and female flowers are found on separate plants they are hand-pollinated. The fruits mature and are harvested between September and December. All harvested fruit is inspected prior to purchase by BioVittoria. Fruit that is not to be processed immediately is placed in a controlled atmosphere cool store (0-5°C).

1.5.4. Processing of Luo Han Fruit Concentrate

Processing methods are generally similar to those used to produce a number of other fruit-derived products. The fresh fruit is mechanically crushed or shredded. Macerated fruit is decocted for 30-40 minutes at 80°C with deionised water.. The supernatant is allowed to cool to 50°C and is then clarified by passing through an ultrafiltration membrane to remove the large molecules of protein and pectin from the supernatant. The supernatant is then passed through a pressurised resin-packed column. The resin is a divinylbenzene copolymer, a macroporous polymeric adsorbent which removes organic substances from aqueous flows. The resin achieves its effect by electrostatic site-specific attraction, binding the target compounds, principally mogrosides, while allowing unwanted compounds, including remaining traces of

[1] ChromaDex has developed and validated HPLC methods for the analysis of several mogrosides; these methods were used to determine the composition of PureLo® brand Luo Han fruit concentrate.

reducing sugars and mineral salts, to pass through into the waste stream. The action of the resin is mechanical rather than chemical and can best be compared to sieving or straining. Supernatant is continuously introduced into the columns until the binding surface of the resin (approximately 100-1000 m^2/g) is fully saturated.

After the mechanical separation of components of the supernatant has been effected by the resin, the adhered material is released from the resin by elution with successive increments of food-grade aqueous ethanol solution. This process frees virtually all of the adsorbed material from the resin. The ethanol solution does not chemically change any of the compounds but merely brings them into solution. The eluent is heated to approximately 60°C and placed under partial vacuum, allowing the ethanol and bound water vapor to be condensed and recycled. The mother liquor is then cooled to approximately ambient temperature. It is then subjected to a decolorizing step to separate the terpene glycosides in the solution from the melanoidins and other non-terpene glycoside molecules. This is achieved by contacting the mother liquor with a styrene divinylbenzene resin that adsorbs the colored melanoidin compounds and other non-terpene glycoside molecules in the solution. The decolorized mother liquor is then concentrated to approximately 40% soluble solids and spray-dried at 120°C in enclosed conditions, removing any remaining water and ethanol.

After cooling to ambient temperature, the powder is sampled for analysis and sealed in Mylar-coated aluminum bags.

At the end of each run, the resin is regenerated by flushing with a food-grade 0.2% solution of calcium hydroxide, followed by filtered water. Next, a food-grade 0.2% solution of hydrochloric acid is introduced to restore the neutral pH of the resin. Finally, the column is flushed with filtered water.

The steps in the production of Luo Han fruit concentrate from Luo Han fruit are shown schematically in Figure 4.

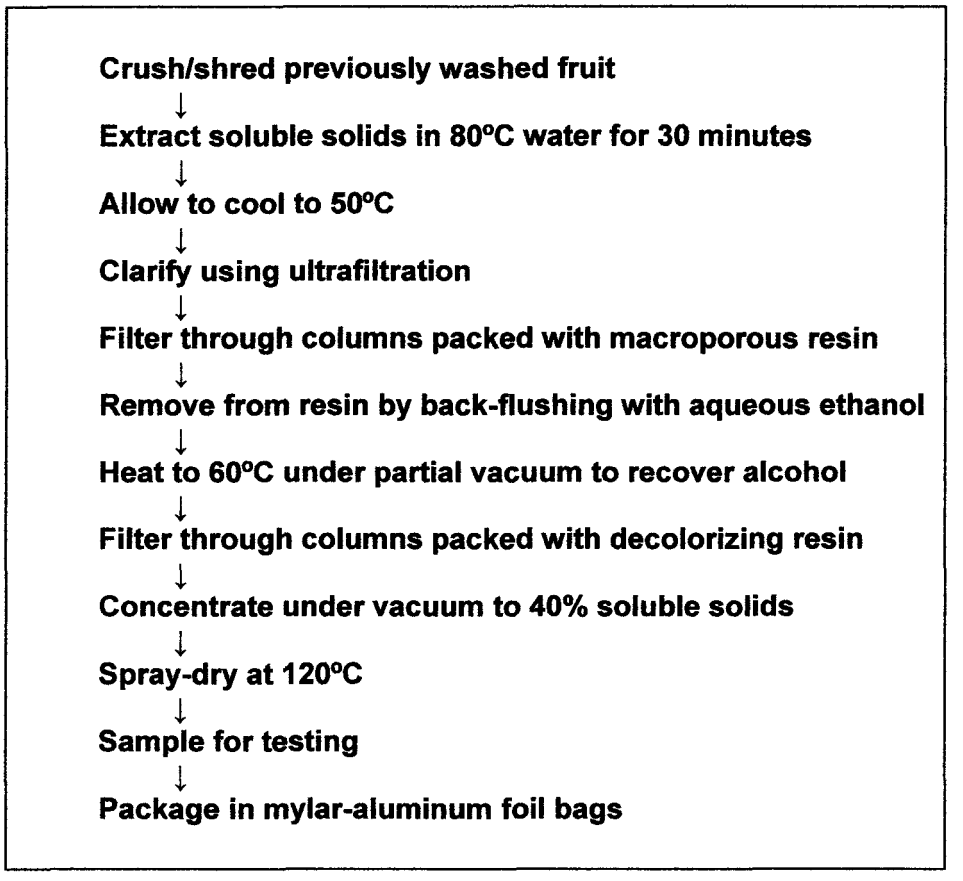

Figure 4. Production Steps for Luo Han Fruit Concentrate.

1.5.5. Regulatory Status of Processing Aids

Divinylbenzene copolymer is approved (21 CFR §173.65) as a secondary direct additive for the removal of organic substances from non-alcoholic aqueous foods, subject only to the requirement that the temperature of the food stream contacting the polymer not exceed 79.4°C. Food-grade ethanol is an unlisted GRAS substance widely used as a solvent in food processing. Food-grade calcium hydroxide and hydrochloric acid, used to regenerate the resin, are both GRAS substances (21 CFR §184.1205 and §182.1057, respectively) with use limited only by current good manufacturing practice (cGMP). Styrene divinylbenzene ion exchange resin is approved under 21 CFR §173.25.

1.6. Product Characteristics of Luo Han Fruit Concentrate

1.6.1. Composition of Luo Han Fruit Concentrate

The typical proximate composition of Luo Han fruit concentrate is shown in Table 2. As discussed above, in UV analyses flavonoids and melanoidins absorb strongly at 600 nm and thus are indistinguishable from mogrosides using this method (Lyndon

2006)[1]. Since UV analyses of PureLo® had indicated a mogroside content of about 69%, while more valid methods indicate about 48%, it may be inferred that flavonoids and melanoidins constitute about 21% of the product. Unfortunately, analytical methods do not exist to confirm this inference, but, if correct, this accounts for nearly all of the 24% unidentified constituents in the results shown in Table 2.

Scientists at Bucher-Alimentech developed an HPLC method for estimating the total mogroside content of Luo Han fruit concentrate by integrating all of the peaks in the HPLC trace that are identified as providing photodiode fingerprints consistent with being triterpene glycosides (Lyndon 2006). This method, which has not been validated, assumes that all mogrosides have the same absorbance as mogroside V. Using this method, Luo Han fruit concentrate is characterized as comprising about 35% mogroside V and about 48% total mogrosides. The HPLC chromatographs indicate that the remaining 24% of the material, which was identified as mogroside with UV-Vis spectroscopy analyses, produces peaks consistent with its being approximately half flavonoids and half melanoidins.

Table 2. Proximate Composition of Luo Han Fruit Concentrate.

Component	Concentration (%)	Analytical Method[1]
Mogrosides		Liquid Chromatography/ Mass Spectrometry
Mogroside V	34.90	
11-Oxo-Mogroside V	8.01	
Grosmomoside I	2.67	
Siamenoside I	1.95	
Sucrose	2.78	Gas chromatography
Fiber	0.10	AOAC 991.43
Ash	1.57	Residue on ignition
Free fatty acids	0.01	Gas chromatography
Water	2.46	Loss on drying
Protein fragments[2]	21.10	Bicinchoninic Acid

1. Descriptions of the analytical methods are provided in Appendix I; ChromaDex reports are in Appendix II
2. Molecular weights < 100 kD

1.6.2. Specifications for the Food-Grade Material

BioVittoria has developed specifications for PureLo® Luo Han fruit concentrate to ensure that it is a safe and wholesome food suitable for human consumption. These specifications are listed in Table 3, along with the reference to the BioVittoria standard operating procedure (SOP) for determining compliance with each specification.

[1] Several published studies (e.g., Song et al. 2006 and 2007) report 80% mogroside content in water extracts of Luo Han fruit and are almost certainly inadvertently including flavonoids and melanoidins.

Table 3. Food Grade Specifications for PureLo® Luo Han Fruit Concentrate.

Parameter	Specification	Test Method
Assay: Mogroside V	≥ 30%	HPLC
Color	Light yellow	GB/T[1] 5492-2008
Odor	Mild fruity; characteristic	GB/T 5492-2008
Taste	Sweet	GB/T 5492-2008
Identification	Positive	TLC
pH	6.0 ± 0.5	
Ash	≤ 5.0%	AOAC 942.05, 17th
Mesh Size	95% through 80 mesh	80 mesh screen
Bulk Density	0.450 – 0.600 g/ml	Densitometer
Solubility	Fully soluble in water	NLS 02.65.00
Method of Extraction	Water	
Extract Solvents	Water	
Moisture	≤ 6.0%	GB/T 12531-1990
Heavy Metal	≤ 20 mg/kg	USP 24 mono (231)
Arsenic (As)	≤ 0.05 mg/kg	GB/T 5009 11-2003
Cadmium (Cd)	≤ 1 mg/kg	GB/T 5009 12-2003
Lead (Pb)	≤ 1 mg/kg	GB/T 5009 12-2003
Phosphate Organics	≤ 1 mg/kg	Gas Chromatography
Organic Residues	≤ 1 mg/kg	Gas Chromatography
Pesticide Residues	≤ 1 mg/kg	Gas Chromatography
Aerobic Plate Count	≤ 10,000 cfu[2]/g	GB/T 4789 2-2008
Total Yeast & Mold	≤ 100 cfu/g	GB/T 4789 15-2003
E. Coli	Negative in 25 g	GB/T 4789 3-2008
Salmonella	Negative in 25 g	GB/T 4789 4-2008
Staphylococcus	Negative in 25 g	

1. GB/T = recommended Chinese National Standard
2. Colony-forming units

1.6.3. Batch Analysis Results

To demonstrate conformance with the specifications listed above, BioVittoria analyzed 5 nonconsecutive lots of its final material. The results of these analyses are displayed in Table 4. These results show that all 5 lots of Luo Han fruit concentrate are in full compliance with the established specifications, and thus the production process is under control and capable of consistently producing food-grade product. In addition to the analyses shown in Table 4, BioVittoria commissioned heavy-metal analyses of a

single lot of Luo Han fruit concentrate with lower limits of detection. These analyses found lead at 0.26 ppm, cadmium at 0.011 ppm, mercury at 0.01 ppm, copper at 0.36 ppm, and arsenic at 0.1 ppm—all well within specification.

Table 4. Results of Analyses of 5 Lots of PureLo® Luo Han Fruit Concentrate.

Parameter	Specification	Lot Number				
		S2005-0105	S2005-0112	S2004-1130	S2005-0108	S2004-1116
Mogroside V	≥ 30%	30.42%	30.17%	30.14%	30.25%	30.49%
Organoleptic Characteristics						
Appearance	Light yellow powder	Meets	Meets	Meets	Meets	Meets
Odor	Mild fruity	Meets	Meets	Meets	Meets	Meets
Taste	Sweet	Meets	Meets	Meets	Meets	Meets
Physical Characteristics						
Particle size	≥ 95% pass 80 mesh	100%	100%	100%	100%	100%
Moisture	≤ 6.0%	4.38%	4.09%	4.12%	3.97%	3.84%
Solubility in water	Dissolves easily	Meets	Meets	Meets	Meets	Meets
Heavy Metals						
Arsenic	≤ 0.5 mg/kg	Meets	Meets	Meets	Meets	Meets
Lead	≤ 1.0 mg/kg	Meets	Meets	Meets	Meets	Meets
Copper	≤ 5.0 mg/kg	Meets	Meets	Meets	Meets	Meets
Microbiology						
Total plate count	< 10,000/g	Meets	Meets	Meets	Meets	Meets
Total yeast & mold	< 100/g	Meets	Meets	Meets	Meets	Meets
E. coli	Negative in 25 g	Meets	Meets	Meets	Meets	Meets
Total pathogens	Negative in 25 g	Meets	Meets	Meets	Meets	Meets
Source: BioVittoria Ltd.						

1.6.4. Pesticide Residue Analyses

Extensive analyses with low limits of detection (LOD) were conducted with a single lot of Luo Han fruit concentrate to demonstrate the absence of detectable pesticide residues; as shown in Table 5, no pesticide residues were found. Since no pesticides are used in the production of the product, this was an expected finding.

Table 5. Limits of Detection of Pesticide Analyses of PureLo® Luo Han Fruit Concentrate.

Compound	LOD* mg/kg	Compound	LOD* mg/kg	Compound	LOD* mg/kg
Acephate	0.08	Chlorpyrifos methyl	0.04	Endosulphan II	0.02
Acetochlor	0.04	Chlorthal-dimethyl	0.04	Endosulphan sulfate	0.02
Alachlor	0.04	Chlortoluron	0.04	Endrin	0.02
Aldrin	0.02	Chlozolinate	0.04	Endrin Aldehyde	0.02
Atrazine	0.04	Clomazone	0.08	Endrin Ketone	0.02
Atrazine-desethyl	0.04	Coumaphos	0.08	EPN	0.04
Atrazine-desisopropyl	0.12	Cyanazine	0.04	Epoxiconazole	0.08
Azaconazole	0.04	Cyanophos	0.04	EPTC	0.04
Azinphos methyl	0.08	Cyfluthrin	0.04	Esfenvalerate	0.04
Azoxystrobin	0.08	Cyhalothrin	0.04	Esprocarb	0.08
Benalaxyl	0.04	Cypermethrin	0.04	Ethion	0.04
Bendiocarb	0.04	Cyproconazole	0.04	Ethoprophos	0.04
Benodanil	0.04	Cyprodinil	0.08	Etridiazole	0.08
BHC (alpha)	0.02	DDD (2,4')	0.02	Etrimphos	0.04
BHC (beta)	0.02	DDD (4,4')	0.02	Famphur	0.04
BHC (delta)	0.02	DDE (2,4')	0.02	Fenamiphos	0.04
Bifenox	0.04	DDE (4,4')	0.02	Fenarimol	0.04
Bifenthrin	0.04	DDT (2,4')	0.02	Fenchlorphos	0.04
Bitertanol	0.04	DDT (4,4')	0.02	Fenitrothion	0.04
Bromacil	0.04	Deltamethrin	0.04	Fenobucarb	0.08
Bromophos ethyl	0.04	Demeton-s-methyl	0.12	Fenoxaprop-ethyl	0.08
Bromopropylate	0.04	Diazinon	0.04	Fenpiclonil	0.04
Bupirimate	0.04	Dichlobenil	0.04	Fenpropathrin	0.04
Buprofezin	0.04	Dichlofenthion	0.04	Fenpropimorph	0.04
Butamifos	0.04	Dichlofluanid	0.04	Fensulfothion	0.04
Cadusafos	0.04	Dichloran	0.04	Fenthion	0.04
Captafol	0.04	Dichlorvos	0.08	Fenvalerate	0.04
Captan	0.04	Dicofol	0.20	Fluazifop-butyl	0.04
Carbaryl	0.08	Dicrotophos	0.04	Flucythrinate	0.04
Carbofenothion	0.04	Dieldrin	0.02	Fludioxonil	0.08
Carbofuran	0.04	Difenoconazole	0.04	Fluometuron	0.04
Carboxin	0.04	Diflufenican	0.04	Flusilazole	0.08
Chlordane, cis-	0.02	Dimethenamid	0.04	Flutriafol	0.08
Chlordane, trans-	0.02	Dimethoate	0.08	Fluvalinate	0.04
Chlorfenvinphos (E+Z)	0.04	Dimethylvinphos	0.04	Folpet	0.04
Chlorfluazuron	0.04	Dinocap	0.20	Furalaxyl	0.04
Chlorobenzilate	0.04	Diphenylamine	0.08	Furathiocarb	0.08
Chlorothalonil	0.04	Disulfoton	0.20	Halfenprox	0.04
Chlorphenapyr	0.04	Diuron	0.08	Haloxyfop-methyl	0.04
Chlorpropham	0.04	Edifenphos	0.04	HCB	0.02
Chlorpyrifos	0.04	Endosulphan I	0.02	Heptachlor	0.02

* LOD = Limit of Detection; no pesticide residues were detected

Table 5. Limits of Detection of Pesticide Analyses, cont.

Compound	LOD* mg/kg	Compound	LOD* mg/kg	Compound	LOD* mg/kg
Heptachlor Epoxide	0.02	Omethoate	0.12	Pyrimethanil	0.04
Hexaconazole	0.04	Oxadiazon	0.04	Pyriproxyfen	0.08
Hexazinone	0.04	Oxadixyl	0.04	Quinalphos	0.04
Hexythiazox	0.12	Oxychlordane	0.02	Quintozene	0.04
Imazalil	0.12	Oxyfluorfen	0.04	Quizalofop-ethyl	0.04
Indoxacarb	0.04	Paclobutrazol	0.04	Simazine	0.04
Iodofenphos	0.04	Parathion ethyl	0.04	Simetryn	0.08
Iprodione	0.04	Parathion methyl	0.04	Sulfentrazone	0.04
Isazophos	0.04	Penconazol	0.04	Sulfotep	0.04
Isofenphos	0.04	Pendamethalin	0.04	Tebufenpyrad	0.04
Isoprocarb	0.08	Phosalone	0.08	Terbacil	0.04
Kresoxim methyl	0.04	Phosmet	0.04	Tebuconazole	0.04
Leptophos	0.04	Phosphamidon	0.04	Terbufos	0.04
Lindane (gamma-BHC)	0.02	Pirimicarb	0.04	Terbumeton	0.04
Linuron	0.20	Pirimiphos methyl	0.04	Terbuthylazine	0.04
Malathion	0.04	Prochloraz	0.04	Terbuthylazine desethyl	0.04
Metalaxyl	0.08	Procymidone	0.04	Terbutryn	0.08
Methacrifos	0.04	Profenofos	0.04	Tetrachlorvinphos	0.04
Methamidophos	0.08	Prometryn	0.04	Tetradifon	0.04
Methidathion	0.04	Propachlor	0.08	Thenylchlor	0.04
Methiocarb	0.08	Propaphos	0.04	Thiobencarb	0.04
Methoxychlor	0.02	Propazine	0.04	Thiometon	0.08
Metolachlor	0.04	Propetamphos	0.04	Tolclofos-methyl	0.04
Metribuzin	0.04	Propham	0.04	Tolylfluanid	0.04
Mevinphos	0.04	Propiconazole	0.04	Triadimefon	0.04
Monocrotophos	0.04	Propoxur	0.08	Tri-allate	0.08
Myclobutanil	0.04	Propyzamide	0.04	Triazophos	0.04
Naled	0.12	Pyraclofos	0.08	Trifloxystrobin	0.08
Napropamide	0.08	Pyrazophos	0.04	Trifluralin	0.04
Nitrofen	0.04	Pyrazoxyfen	0.08	Vinclozolin	0.08
Nitrothal-isopropyl	0.04	Pyrethrin	0.04		
Norflurazon	0.04	Pyrifenox	0.04		

* LOD = Limit of Detection; no pesticide residues were detected

1.6.5. Inter-Lot Compositional Consistency of PureLo® Luo Han Fruit Concentrate

Four lots of Luo Han fruit concentrate were analyzed by HPLC. These traces are overlaid in Figure 5 to demonstrate the consistency of composition across lots of product.

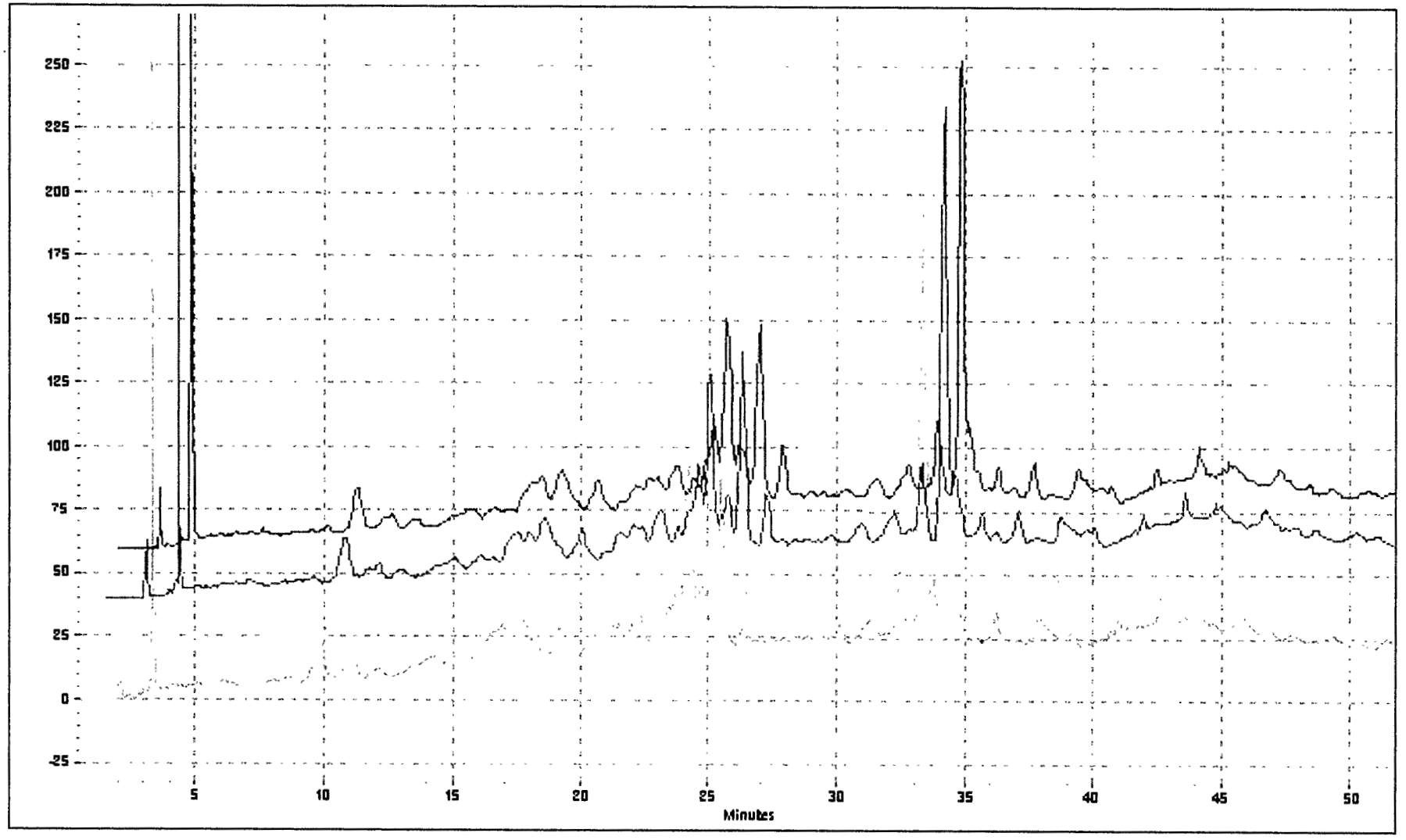

Figure 5. HPLC Traces of 4 Lots of PureLo® Luo Han Fruit Concentrate.

1.7. Stability

The stability of Luo Han fruit concentrate in a dietary matrix was assessed both in an open container at room temperature and in an airtight container stored in a refrigerator (Sheehy 2007). The chosen dietary matrix was AIN-93G Rodent Diet, with Luo Han fruit concentrate added at concentrations of 0, 1, 3, and 10%. The primary endpoint was the mogroside V content as measured by HPLC/UV. The room-temperature samples were analyzed in duplicate at 0, 7, 10, and 14 days; refrigerated samples were analyzed in duplicate on days 0 and 7. The results are summarized in Table 6. As is clear, the HPLC/UV analyses showed only analytical variability and no clear time trend.

Table 6. Results of Luo Han Fruit Concentrate Stability Study.

Storage Condition and Day	PureLo® Concentration		
	1%	3%	10%
	Mogroside V Content (% of Day 0 Level)		
Room Temperature			
Day 7	112.5	97.5	102.9
Day 10	90.4	83.2	106.8
Day 17	93.3	83.3	114.1
Refrigerated			
Day 7	99.8	97.1	100.8
Source: Sheehy 2007			

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2. INTENDED TECHNICAL EFFECT

The intended technical effect of the addition of Luo Han fruit concentrate to foods is as a non-nutritive sweetener [21 CFR §170.3(o)(19)] and flavor enhancer [21 CFR §170.3(o)(11)]. Unlike most other non-nutritive sweeteners, Luo Han fruit concentrate is a traditional food that has been consumed and added to other foods for more than a century in the U.S. and elsewhere for its sweetness. The intended use of Luo Han fruit concentrate is as a stand-alone sweetener, a food ingredient, and a component of sweetener blends that would include other permitted sweeteners.

As noted by Song et al. (2007) and Matsumoto et al. (2009), the triterpene glycosides in Luo Han contain sapogenin with a triterpenol structure and the glucosidic bonds are in β configuration, and so they are not decomposed and digested by amylase in humans and cannot be much absorbed and converted into energy. (Additionally, as determined by Gibson [2007], discussed later, there appears to be little if any fermentation of Luo Han extract by colonic bacteria.)

The sweetness intensity of Luo Han extracts, as compared to sucrose, has been variously estimated as 150 (Lee 1975), 256 (Kinghorn 1987), and 300 (Yoshikawa et al. 2005). A licorice-like taste at high levels of intake has been noted by several investigators. Matsumoto et al. (1990) had expert tasters evaluate the sweetness of isolated glycosides. Mogrosides V and IV were judged to be 425 and 392 times sweeter than sucrose, respectively, while 11-oxo-mogroside V was rated as 84 times sweeter than sucrose and siamenoside I was 563 times sweeter. The same tasters found that mogroside III was tasteless. Yoshikawa et al. (2005) assessed the sweetness of mogroside V as 378 times that of sucrose.

RSSL LinTech was contracted by BioVittoria to evaluate the sweetness characteristics of PureLo® Luo Han fruit concentrate (RSSL LinTech 2007). The objectives were to determine the sweetness potency of this specific fruit concentrate and to evaluate other characteristics such as sweetness quality and sweetness stability in selected food products.

Three samples of Luo Han fruit concentrate, with mogroside V contents ranging from 33% to 46%, were provided; most of the testing used the highest mogroside V content sample. Sucrose solutions were prepared both in commercial bottled water (pH 7.0 to 7.5) and in citric acid/trisodium citrate buffer (pH 3.0), with concentrations of 2%, 5%, and 8% (w/v). A panel of 8 experienced tasters was presented with one of the sucrose solutions and a series of Luo Han fruit concentrate test solutions increasing in concentration, and asked to identify the Luo Han fruit concentrate solution most closely matching the sweetness of the sucrose control.

In a paired-comparison test (referred to as Beck's methodology), 20 tasters were give one sample of sucrose solution and one sample of Luo Han fruit concentrate solution and asked to report which solution tasted sweeter. The proportion of tasters evaluating each test as sweeter than the reference was plotted against Luo Han fruit concentrate

concentration in order to estimate a concentration at which 50% of the tasters perceived the Luo Han fruit concentrate solution as sweeter, referred to as the iso-sweet point.

The results of the first test indicated sweetness factors for Luo Han fruit concentrate (in sucrose equivalents) of 100-182x against the acidic buffer solution and 100-208x against the bottled water solution. The paired-comparison method found sweetness potency ranges of 74-123x and 94-179x against the two types of solution, respectively. The authors concluded that Luo Han fruit concentrate is sweeter relative to sucrose at pH 7 than at pH 3. The overall average sweetness potency of the 33% mogroside V sample of PureLo® Luo Han fruit concentrate was estimated as about 95x sucrose, but potentially higher in neutral pH matrices.

3. INTENDED USE AND CONSUMER EXPOSURE

Luo Han fruit concentrate is intended to be used as a stand-alone sweetener or a food ingredient, and as a component of sweetener blends that can be added to foods or used as tabletop sweeteners. Because Luo Han fruit concentrate is much sweeter than sucrose, the amount that is needed to obtain the same degree of sweetness is much less. Further, as noted in several published reports, at high concentrations Luo Han fruit concentrate and other Luo Han extracts appear to provide a "licorice" aftertaste that may not be desirable in many applications and which may limit its use.

At this time, the market for intense sweeteners in the U.S. may be regarded as mature. With saccharin, aspartame, sucralose, acesulfame, and alitame all available, market niches for intense sweeteners have been filled. This means that a new sweetener is not competing with sucrose, but with existing intense sweeteners. Thus, the appropriate method for estimating potential intake of Luo Han fruit concentrate is to determine existing levels of intake of intense sweeteners, convert these intakes into sucrose equivalents in order to establish a common metric, and then determine the amount of Luo Han fruit concentrate needed to replace this amount of sucrose equivalence.

An assessment of intense sweetener intake, including conversion of these intakes to sucrose equivalents, was recently completed by Renwick (2008) in order to predict dietary exposures for the intense sweetener rebaudioside A[1]. Published data on intakes of intense sweeteners were collected from a large number of countries, including the U.S., Canada, the UK, Germany, Denmark, Netherlands, France, Australia, and New Zealand. These data were converted to sucrose equivalents using the following estimates of sweetness relative to sucrose: saccharin = 300, aspartame = 180, sucralose = 600, acesulfame = 200, alitame - = 2000, and cyclamates (not available in the U.S.) = 30.

Renwick (2008) provided estimates of both mean and 90th percentile intakes of intense sweeteners, in sucrose equivalents, for the general population, diabetic adults, healthy children, and diabetic children. These data are presented in Table 7. Also shown in the table are the amounts of Luo Han fruit concentrate needed to replace these intense sweeteners assuming a relative sweetness of 100.

The figures shown in Table 7, of course, represent extremely conservative estimates of the potential intake of Luo Han fruit concentrate since they assume that this sweetener will capture the entire intense-sweetener market. For the general population, the estimated maximum mean intake of Luo Han fruit concentrate is 2.6 mg/kg bw/day and the 90th percentile is 6.8 mg/kg bw/day. Potential intakes of children are slightly higher, as shown in Table 7.

[1] Renwick (2008) notes that FDA has used a similar method to predict the intakes of acesulfame-K and sucralose.

Table 7. Current Daily Intake of Intense Sweeteners (In Sucrose Equivalents) and Estimated Daily Intakes of Luo Han Fruit Concentrate.

Population Group	Intakes of Intense Sweeteners (mg sucrose/kg bw/day)*		Intake of Luo Han Fruit Concentrate To Replace All Intense Sweeteners (mg/kg bw/day)	
	Mean	90th Percentile	Mean	90th Percentile
General population	255	675	2.6	6.8
Diabetic adults	280	897	2.8	9.0
Healthy children	425	990	4.2	9.9
Diabetic children	672	908	6.7	9.1
*Source: Renwick (2008)				

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4. REVIEW OF SAFETY DATA

This GRAS determination is based on scientific procedures, but it should be recognized that Luo Han fruit concentrate could most likely be regarded as GRAS under its intended conditions of use through experience based on the common use of Luo Han water extracts in food. Even in a scientific-procedures GRAS assessment, this history of common use in food contributes to the evidence demonstrating safety. Luo Han's history of use includes centuries of widespread use of Luo Han fruit and fruit concentrates in China, more than a century of documented use of Luo Han fruit in the U.S., and more recent common use in the U.S. and worldwide of a variety of sweeteners derived from Luo Han.

4.1. Toxicity Studies of Luo Han Guo

4.1.1. Acute Oral Toxicity

Male albino mice weighing 19-24 g were dosed via gavage with aqueous solutions of lyophilized Luo Han extracts at a volume of 0.03 ml/g bw, providing doses as high as 15,000 mg extract/kg bw (Lee 1975). Both crude and refined extracts were tested with n=10 animals/group; animals were observed for 7 days after dosing. There was no mortality. At the maximum dose of 15,000 mg/kg bw the animals exhibited mild transient sedation and some diarrhea, but these effects disappeared within 30-60 minutes. Lee (1975) reported the LD_{50} for mice as being in excess of 10,000 mg/kg bw.

Makapugay et al. (1985) reported that a Luo Han extract tested in their laboratory produced no mortality in acute toxicity experiments on mice at doses up to 2000 mg/kg bw, establishing an LD_{50} greater than this dose. (They also reported that the extract was nonmutagenic, but no further information was provided regarding these studies.)

Hussain et al. (1990) orally administered doses of 0, 1000, or 2000 mg Luo Han extract/kg bw by gavage to 4-6-week-old male Swiss-Webster mice and observed them for 14 days. No changes in body weight or other indications of toxicity were seen at the doses tested, and the LD_{50} in this study was >2000 mg/kg bw.

4.1.2. Subacute Oral Toxicity

A Redbook- and OECD-compliant 28-day dietary study was conducted in Hsd:SD® rats to evaluate the oral toxicity of PureLo® Luo Han fruit concentrate (Marone et al. 2007). Groups of 20 rats (10/sex/group) were fed diets containing 0, 10,000, 30,000, or 100,000 mg Luo Han fruit concentrate/kg feed. One hundred and four rats were obtained at age 6-7 weeks and held for an 8-day acclimation period before being assigned to test groups based on having body weights within ±20% of the mean group weight. At study initiation, the male rats weighed 203-221 g (mean = 211.6 g) and the females 151-169 g (mean = 159.5 g). The animals were individually housed in wire mesh cages in a climate-controlled environment and received filtered tap water *ad libitum*. During the acclimation period the rats were fed *ad libitum* AIN-93G Rodent Diet; test diets were formulated weekly by adding Luo Han fruit concentrate and sucrose to achieve target concentrations while providing equal nutrient content and near isocaloricity. The caloric density of the control and low-dose diets was 4.0 kcal/g, that of

the mid-dose diet was 3.9 kcal/g, and that of the high-dose diet was 3.6 kcal/g. Diet samples were tested periodically to evaluate concentration and homogeneity.

Rats were fed *ad libitum* for 28 days, and feed consumption and body weight were measured weekly; feed efficiency was calculated as mean daily body weight gain divided by mean daily feed consumption. All animals were observed twice daily for signs of toxicity, morbidity, and mortality. Detailed clinical observations (skin, fur, eyes, mucous membranes, secretions and excretions, autonomic activity, gait, clonic or tonic movements, grooming, repetitive circling, response to handling, or abnormal behavior) were made prior to study initiation and weekly during the study period. Prior to study initiation and on day 23 the eyes of all animals were examined by focal illumination, indirect ophthalmoscopy, and, when needed, slit-lamp microscopy.

At least one day before clinical pathology evaluation, animals were fasted for 15 hours and placed in metabolism cages; urine was collected and analyzed for volume, quality, clarity, color, pH, ketone, glucose, bilirubin, urobilinogen, protein, specific gravity, blood, and microscopic sediment. Animals were fasted overnight and blood samples were collected via orbital sinus bleeding and via the inferior vena cava prior to terminal sacrifice on day 29 (males) or day 30 (females). Hematology parameters were erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, red cell distribution width, absolute reticulocyte count, platelet count, total white blood cell, and differential leukocyte count. Clinical chemistry parameters included serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), sorbitol dehydrogenase, alkaline phosphatase (ALP), total bilirubin, urea nitrogen, blood creatinine, total cholesterol, triacylglycerols, fasting glucose, total serum protein, albumin, globulin, calcium, inorganic phosphorus, sodium, potassium, and chloride. Serum samples from 2 randomly chosen animals were pooled and a viral screen was performed.

All animals were weighed and euthanized by exsanguination. The liver, kidneys, adrenals, brain, heart, thymus, spleen, testes, epididymides, uterus and ovaries were removed and weighed. Histological examinations were performed on the following organs and tissues from the control and high-dose groups: lungs, trachea, brain (including sections of the medulla/pons, cerebellar cortex and cerebral cortex), spinal cord (3 levels: cervical, mid-thoracic, and lumbar), salivary glands, thymus, heart, sternum with bone marrow, adrenals, liver, spleen, kidneys, thyroid/parathyroid, urinary bladder, ovaries and fallopian tubes, uterus, vagina, esophagus, ileum, cecum, accessory genital organs (prostate and seminal vesicles), peripheral nerve (sciatic), stomach, duodenum, jejunum, colon, rectum, representative lymph node (mesenteric and mandibular), pancreas, pituitary gland, aorta, female mammary gland, Harderian gland, skin, nasal turbinates, and skeletal muscle.

No mortality occurred and no adverse clinical findings were observed. Both eyes of all study animals were ophthalmoscopically normal. Statistically significant decrements in body weights were observed in both sexes at the highest dietary concentration of Luo Han fruit concentrate during several weekly intervals and for the overall in-life duration of the study. Feed consumption paralleled the body weight results; statistically significantly reduced feed intake was noted during several weeks for both

sexes in the high-dose group. Feed efficiencies were similar for all dietary concentrations except high-dose males during week 1.

The mean overall daily intakes of Luo Han fruit concentrate in rats fed concentrations of 0, 10,000, 30,000, or 100,000 ppm were 0, 733, 2096, and 7071 mg/kg bw/day for males and 0, 743, 2147, and 7478 mg/kg bw/day for females. A few statistically significant differences in hematological measures were noted (increased hemoglobin and hematocrit and decreased white blood cell and lymphocyte counts in high-dose males, increased red blood cell hemoglobin concentration in mid-dose females, decreased prothrombin time in mid- and high-dose females), but were not considered adverse since they were slight, occurred in only one sex, and/or were not dose-related. Additionally, although the red cell mass parameters of hemoglobin and hematocrit were slightly increased in high-dose males compared to controls, there was no apparent change in red cell morphology. The statistical significance of the decreased prothrombin time in females was due primarily to one low value in the high-dose group.

Similarly, the clinical chemistries exhibited a few differences not regarded as toxicologically significant since they lacked histopathologic correlates and were seen in only one sex or were not dose-related; observed differences were slightly decreased bilirubin in low-dose females and in mid- and high-dose groups of both sexes, slightly increased total protein due to albumin in low- and high-dose males or globulin in mid- and high-dose females, and increased potassium and slightly decreased chloride in high-dose females.

No significant treatment-related findings were seen in the urinalysis, and no detectable titers were seen in the serological study against the pathogens and antigens tested. The gross necropsy revealed no abnormalities attributable to Luo Han fruit concentrate administration; incidental findings included fluid-filled uteri in some females from all groups, larger than normal adrenal glands with no histopathologic correlate in one high-dose female, and a moderate-grade spermatic granuloma in one low-dose male. There were no statistically significant changes in absolute organ weights except increased liver weight in high-dose females. Relative weights of liver, adrenals, testes, and epididymides in high-dose males were significantly elevated, while females showed statistically significant increases in relative liver weights in all dose groups and increases in ovary relative weights in the low- and high-dose groups. The lack of histopathological hepatic findings or changes in liver enzymes suggests that liver weight changes are of limited toxicological interest. No treatment-related microscopic changes were reported, although retrobulbar and Harderian gland inflammation was frequently observed in both sexes in both the control and high-dose groups.

It was concluded that the reductions seen in feed consumption and consequent body weight gain may have been due to the additional bulk of the test substance in the diet, which was at a maximum concentration of 10%. Additionally, Luo Han fruit concentrate, like other extremely sweet substances, is aversive at high concentrations and may have affected the palatability of the diets. The similarity of feed efficiencies at all dietary concentrations indicates that the reduced body weights observed were due to reduced feed consumption and not toxicity. The NOAEL in this study (Marone et al.

2007) was determined to be the highest dietary concentration tested, providing doses of 7071 and 7478 mg/kg bw/day for male and female rats, respectively.

Three 4-week and one 8-week feeding studies conducted by the same laboratory in alloxan-induced diabetic mice were designed primarily as nutrition studies rather than safety studies, but included a number of measures of toxicity endpoints (Qi et al. 2006; Song et al. 2006; Song et al. 2007; Qi et al. 2008). Two of the studies (Qi et al. 2006 and 2008) tested an ethanol extract of Luo Han while the other two (Song et al. 2006 and 2007) used a water extract as the test article.

Qi et al. (2006) compared the effects of an ethanol extract of Luo Han on the splenic lymphocyte and cytokine expression levels of normal mice and alloxan-induced diabetic mice. Fresh Luo Han fruits were extracted with 70% aqueous ethanol, concentrated, and dried to produce a powder. Male Balb/c mice weighing 18-20 g (number, age, and caging were not reported) were acclimated for a week, after which half of them were fasted for 18 hours and injected intraperitoneally with alloxan to induce diabetes, which was confirmed by measurement of blood glucose level. Both normal and diabetic mice were divided into 3 groups: 1) control mice, which received distilled water by gavage; 2) low-dose group, gavaged with 150 mg extract/kg bw/day; 3) high-dose group, gavaged with 300 mg extract/kg bw/day. Treatment continued for 4 weeks; feed and water were available *ad libitum*, and feed and water intake were measured daily. Body weight was measured weekly. After sacrifice blood samples were taken from the retroorbital plexus for analysis of blood glucose and spleen tissue samples were taken for measurement of T-lymphocytes and enumeration of CD4 and CD8 subpopulations as well as expression of cytokines IFN-γ, TNF-α, and IL-4 in lymphocytes. The pancreas was also removed for histopathological examination.

The alloxan-induced diabetic mice exhibited hyperglycemia and loss of body weight, as well as significantly increased fasting blood glucose as compared with non-diabetic controls. Alloxan caused a significant increase in splenic CD8 lymphocytes, but not CD4 types; the low dose of extract significantly ameliorated this effect although the effect of the high dose was not significant. Expression of all cytokines was significantly raised in the diabetic mice; Luo Han extract significantly reduced the expression of IFN-γ and TNF-α. Administration of the extract also significantly ameliorated the injury seen in the pancreatic tissues of alloxan-induced diabetic mice. Qi et al. (2006) reported that no adverse effects were observed in either diabetic or normal mice. In normal mice, administration of Luo Han extract at either 150 mg or 300 mg/kg bw/day for 4 weeks had no effect on any of the parameters measured, including body weight, blood glucose, T-lymphocytes, cytokine expression, or pancreatic histology.

The work of Song et al. (2006) was generally similar, except that the test article was a water (rather than ethanol) extract of Luo Han. Male Balb/c mice weighing 18-20 g (age and caging were not reported) were acclimated for a week, after which they were fasted for 18 hours and injected intraperitoneally with either saline solution or alloxan to induce diabetes, which was confirmed by measurement of blood glucose level. Both normal and diabetic mice were divided into 3 groups: 1) control mice, which received distilled water by gavage (n = 10 normal, 7 diabetic mice); 2) low-dose group, gavaged

with 150 mg extract/kg bw/day (n = 10 normal, 8 diabetic); 3) high-dose group, gavaged with 300 mg extract/kg bw/day (n = 10 normal, 11 diabetic).

Treatment continued for 30 days; feed and water were available *ad libitum* and feed and water intake were measured daily. Body weight was measured weekly. After sacrifice blood samples were taken from the ocular vein for analysis of blood glucose and insulin, pancreases were removed and subjected to histological examination, and spleens were removed and processed to isolate viable cells. Half of the available aliquots were suspended and incubated with CD4 and CD8 antibodies for 30 minutes. Phenotype analysis of the lymphocytes was performed to determine the percentage of lymphocytes positive for each of the antibodies. The remaining aliquots were cultured for 72 hours and incubated with IFN-γ, TNF-α, and IL-4 antibodies for 30 minutes; the percentages of cells producing IFN-γ, TNF-α, and IL-4 cytokines were calculated.

There were no significant differences in body weights of all 6 groups. Diabetic mice consumed significantly more water and had significantly higher blood glucose and insulin levels; administration of Luo Han extract significantly reduced these effects but did not eliminate them. Administration of the extract effectively regulated the immune imbalance in alloxan-induced mice by up-regulating CD4 T-lymphocyte populations and producing a shift from the expression of pro-inflammatory Th1 cytokines towards a beneficial Th2 pattern in the diabetic mice; differences in normal mice were generally unremarkable. Induction of diabetes resulted in atrophy and degeneration in the islets of Langerhaus, which was significantly reduced by dosing with Luo Han extract. Song et al. (2006) concluded that the tested water extract of Luo Han exhibited no toxicity and had no significant effects on normal mice while attenuating the adverse effects of diabetes.

In a follow-up study (Song et al. 2007), alloxan-induced diabetic mice were used to investigate the effect of the same water extract of Luo Han on renal mitochondrial lipid peroxidation, anti-oxidative defenses, and the oxidative stress-responsive protein heme oxygenase-1 (HO-1). Male Balb/c mice weighing 18-20 g (number, age, and caging were not reported) were acclimated for a week, after which they were fasted for 18 hours and injected intraperitoneally with either saline solution or alloxan to induce diabetes, which was confirmed by measurement of blood glucose level. Both normal and diabetic mice were divided into 3 groups: control mice that received distilled water by gavage, low-dose group gavaged with 150 mg extract/kg bw/day, and high-dose group gavaged with 300 mg extract/kg bw/day.

Treatment continued for either 4 or 8 weeks; feed and water were available *ad libitum* and feed and water intake were measured daily. Body weight was measured weekly. After sacrifice by cervical dislocation blood samples were taken from the ocular vein for analysis of serum glucose, total cholesterol, triacylglycerol, blood urea nitrogen, and creatinine. Kidneys were removed and small sections excised for histopathological examination while the remaining tissue was homogenized and centrifuged to obtain the mitochondrial fraction. which was analyzed for glutathione concentration, manganese superoxide dismutase, and glutathione peroxidase; lipid peroxidation was assayed by measurement of malondialdehyde concentration and HO-1 activity was determined by the generation of bilirubin from heme metabolism.

The alloxan-induced diabetic mice exhibited typical diabetes symptoms, including loss of body weight. Treatment with the low dose of Luo Han extract for 8 weeks ameliorated the polydipsia and polyuria symptoms and significantly increased body weight in diabetic mice. Similarly, the diabetic mice receiving Luo Han extract had glucose levels partially restored to normal after both 4 and 8 weeks and elevated cholesterol and triacylglycerol levels significantly lower after 8 weeks as compared to controls. Diabetic mice showed significantly elevated urea nitrogen and creatinine levels, as well as HO-1 activity and superoxide dismutase after 8 weeks as compared to normal animals, which was partially ameliorated by Luo Han extract. The same pattern appeared with mitochondrial levels of malondialdehyde as a marker for lipid peroxidation. On the contrary, glutathione levels were significantly lowered in diabetic mice, an effect entirely reversed by the tested doses of Luo Han extract. No adverse effects of the extract were reported. Song et al. (2007) concluded that oral exposure to Luo Han water extract had no toxic effect on normal mice but exhibited a beneficial anti-oxidative effect on diabetic mice.

In Qi et al. (2008), the test article was a purified ethanol extract of Luo Han fruit, and the positive control was XiaoKeWann-pill, a compound commonly used in China for the treatment of diabetes. Male Balb/c mice weighing 18-20 g (age not reported) were provided basal diet and water *ad libitum*. Although caging arrangements were not described, it was reported that water consumption was recorded daily. After a 1-week adaptation to the environment and diet, the mice were fasted for 18 hours and then injected with alloxan to induce diabetes, which was confirmed by determination of tail vein blood glucose levels after 3 days. The mice were randomly divided into 7 groups of n = 8 mice/group: 1) non-diabetic control mice, 2) diabetic control mice, 3) diabetic mice gavaged with 50 mg extract/kg bw/day, 4) diabetic mice gavaged with 100 mg/kg bw/day, 5) diabetic mice gavaged with 300 mg/kg bw/day, 6) diabetic mice gavaged with 500 mg/kg bw/day, 7) diabetic mice receiving XiaoKeWann-pill. Treatment continued for 4 weeks, with the mice weighed weekly.

After 4 weeks, blood was taken from the retroorbital plexus following a 12-hour fast, and analyzed for glucose, total cholesterol, HDL-cholesterol, and triacylglycerol concentrations. The liver was removed and activities were measured of glutathione peroxidase, superoxide dismutase, and lipid peroxidation. The alloxan-induced diabetic mice exhibited hyperglycemia and loss of body weight, as well as significantly increased fasting blood glucose as compared with non-diabetic controls. The activities of their liver enzymes were also depressed, and the level of lipid peroxide was raised.

Diabetic mice treated with Luo Han extract gained significantly more weight than did untreated mice, achieving the same weight gain as those receiving XiaoKeWann-pill. They also had significantly decreased total cholesterol and triacylglycerol levels and increased HDL-cholesterol concentrations. Treatment with Luo Han extract significantly reactivated the antioxidant enzymes and reduced levels of lipid peroxide. For all of these endpoints, 50 mg extract/kg bw/day was less effective than were higher levels. Qi et al. (2008) reported no adverse effects as a result of any tested dose of Luo Han extract.

4.1.3. Subchronic Oral Toxicity

A 90-day oral toxicity study in dogs was conducted at Guangxi Normal University, Guilin, Guangxi, in the Peoples Republic of China (PRC) to evaluate any potential subchronic toxicity of PureLo® Luo Han fruit concentrate (Qin et al. 2006). The study was conducted in conformance with PRC guidelines. As allowed in OECD Test Guideline 409 regarding subchronic oral toxicity studies in non-rodents, a limit dose of 3000 mg/kg bw/day was selected as the only test dose.

Five batches of Luo Han fruit concentrate, Control Numbers 951120, 960116, 960206, 960410, and 960518, were provided by the Natural Plant Product Factory of Guilin S&T New Technology Company of Guilin, China. Each batch was tested for uniformity and compliance in providing >80% mogrosides (based on UV analysis). Analytical results fell within target range for all batches.

A cohort of 24 hybrid dogs provided by the animal laboratory of Guilin Hospital, 12 males and 12 females, 8.0 – 9.0 kg, age 24 – 30 weeks, was divided randomly into 4 groups of 6 animals each; 3 animals of each sex were assigned to each dose group. Each group of 6 animals was housed in a separate 40-m^2 room kept at 22 ± 5° C, with 30-40% relative humidity, natural ventilation, and a 12-hour light-dark cycle. Dogs stayed and were fed in-crate, one dog per crate. The crates were 80 cm high, 100 cm in length, and 60 cm in width. The animals were fed 3 times daily with a rice diet supplemented with cooked pork, fish, and vegetables. Distilled water was available *ad libitum*.

Two of the 4 groups, designated LHG I and LHG II, were given a 10 mL/kg bw aqueous solution containing 30% Luo Han fruit concentrate by gavage once per day to provide a dose of 3000 mg/kg bw/day. The 2 remaining groups, Control I and Control II, were given 10 mL/kg bw of distilled water once per day. Animals were dosed for 28 days (LHG I and Control I) or 90 days (LHG II and Control II).

All animals were observed daily for any changes in food and water intake, micturation, stool excretion, activity, and appearance of coat. Dogs in all 4 groups were examined weekly for body weight, heart rate, blood pressure, and respiration. Blood samples were taken at the start of study and then weekly until study conclusion in both the 28- and 90-day LHG and Control animals. Animals were fasted prior to blood sampling. Hematological test parameters were red and white blood cell counts and hemoglobin density. Biochemistry test parameters were albumin, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), fasting glucose, and total serum protein as well as K^+, P^{+++}, Cl^-, and Ca^{++}. Urinalyses were performed at the start of study and then weekly using timed urine volume collection; test parameters included volume, pH, blood urea nitrogen (BUN), creatinine (Cr), glucose, and hemoglobin.

On Day 29, animals in the LHG-I and Control-I groups were euthanized and organs prosected for gross and microscopic pathology. The remaining two groups, LHG II and Control II, were continued on the same dosing regimen for an additional 62 days, for a total of 90 days. On Day 91, animals in these two groups were also euthanized and their organs prosected. Histopathological examinations were conducted of the heart, liver, lungs, kidneys, and spleen.

No unscheduled mortality occurred during the study. Physical observations were generally unremarkable; there were no significant differences in the general condition of the animals. Mean body weights and body-weight gains were similar for the interim LHG-I and Control-I groups and for the final 90-day LHG-II and Control-II groups. No food consumption differences were noted. No significant test-article-related changes in hematology, clinical biochemistry, or urinalysis parameters were noted in either males or females at either 28 days or 90 days. Histopathological examination of major tissues conducted on all animals in all 4 groups revealed no macro- or microscopic lesions attributable to treatment. There were no significant differences in absolute or relative organ weights

Luo Han fruit concentrate was thus well tolerated and did not produce any general organ or systemic toxicity when fed to male and female dogs at a dose of 3000 mg/kg bw/day for up to 90 days. No changes in survival, food consumption, or body-weight gain were found. There were no significant effects on clinical signs or organ weights and no histological changes considered to be related to treatment. There were no adverse or clinically relevant changes in hematology, clinical biochemistry, or urinalysis parameters at either the 28- or 90-day terminal measurement time points. Therefore, the no observed adverse effect level (NOAEL) for PureLo® Luo Han fruit concentrate in this study was the single limit dose tested, 3000 mg/kg bw/day (Qin et al. 2006).

Jin et al. (2007) tested the subchronic oral toxicity of a water extract of Luo Han fruit manufactured by Saraya Co., Ltd., of Japan, with composition nearly identical to PureLo® Luo Han fruit concentrate (including content of 31.4% mogroside V) in Wistar Hannover (GALAS) rats. In the introduction to the article, the authors reported that unpublished contract studies of the Japanese Ministry of Health, Labor and Welfare had found that the LD_{50} of this extract was more than 2000 mg/kg bw when given to rats by gavage; that the extract was not genotoxic in an Ames assay, an *in vitro* chromosome aberration study, or an *in vivo* micronucleus test; that no adverse effects were observed in a 28-day toxicity study in which F344 rats received the extract at 0%, 1%, 2%, or 5% dietary concentration; and that no toxicity was seen in a 90-day study in F344 rats with a maximum Luo Han extract level of 2% dietary concentration.

In the published study (Jin et al. 2007), male and female rats aged 5 weeks and weighing about 115 g (female) or 130 g (male) were housed 2-3 rats per wire-mesh steel cage and given *ad libitum* access to water and to powdered basal diet containing 0%, 0.04%, 0.2%, 1%, or 5% Luo Han extract for 13 weeks. There were 8 rats of each sex in each of the 5 groups. Clinical signs and general appearance were observed daily and food and water consumption and body weights were measured weekly. At the end of the test period, the animals were sacrificed, blood samples were taken from the abdominal aorta, and a necropsy was performed. Hematological parameters included white blood cell count, red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count, as well as ratios of stab cells, segmented neutrophils, eosinophils, lymphocytes, monocytes, and basophils. Clinical chemistries were total protein, albumin, albumin/globulin ratio, total cholesterol, blood urea nitrogen, creatinine, calcium, inorganic phosphate, sodium, potassium, chloride, aspartate aminotransferase, alanine

aminotransferase, and alkaline phosphatase. Weights of brain, heart, lungs, liver, kidneys, spleen, thymus, adrenal glands, pituitary gland, thyroid glands, testes, uterus, and ovaries were measured, and histopathological examinations were conducted for the control and 5% group males and females for these organs as well as the aorta, bone marrow, coagulation gland, esophagus, epididymides, large intestine, lymph node, mammary gland, pancreas, peripheral nerves, prostate gland, salivary gland, skeletal muscle, skin, small intestine, spinal cord, stomach, urinary bladder, tongue, trachea, and vagina.

No deaths nor remarkable changes in general appearance or clinical signs were noted in any group. All groups gained body weight at the same rate and there were no statistically significant differences between groups for either males or females at any time. Similarly, no remarkable differences were observed in average consumption of food or water by the different groups of either sex. The only statistically significant difference in the hematological measures was an increase in the ratios of immature neutrophils and monocytes among males in the 1% and 5% groups. These findings were not regarded as treatment-related because they were small, occurred in only one sex, and were not accompanied by changes in total white blood cell counts. Females in the 5% group showed a significant increase in total cholesterol and decrease in inorganic phosphate. Neither of these changes departed remarkably from the historical range and they were considered to be of no toxicological significance. No other differences were noted in blood biochemistries.

The relative liver weight was significantly increased in males of the 5% group, but the absolute weight was not different and the increased relative weight was within the normal range. In females, both absolute and relative weights of the pituitary glands were significantly increased over the control group, but no histopathological changes were seen and the weight differences were regarded as of no toxicological significance. Since no toxic effects were noted in rats given the test article at 5% in the diet, Jin et al. (2007) determined that the NOAEL in Wistar Hannover rats was 5% dietary concentration, equivalent to 2520 mg/kg bw/day in males and 3200 mg/kg bw/day in females (Jin et al. 2007).

4.1.4. Genetic Toxicity

4.1.4.1. Bacterial Reverse Mutation Tests

The potential for genetic toxicity of a Luo Han extract similar to PureLo® Luo Han fruit concentrate was explored using a standard Ames assay with *Salmonella typhimurium* strain TM 677 in the presence or absence of S9 metabolic activation (Hussain et al. 1990). Five test (mogrosides) groups and one control (distilled water) were assayed. The experiment was conducted with concentrations of 0.31, 0.62, 1.25, 2.5, and 5.0 mg/ml. No bactericidal or genotoxic effect was observed.

PureLo® Luo Han fruit concentrate was subjected to an Ames assay performed in compliance with OECD guideline No. 471, "Bacterial Reverse Mutation Test"; European Commission Regulation No. 440/2008, "Mutagenicity—Reverse Mutation Test Using Bacteria"; and U.S. Environmental Protection Agency Health Effects Test Guideline OPPTS 870.5100, "Bacterial Reverse Mutation Assay" (BSL Bioservice 2009a). PureLo® was tested a concentrations of 31.6, 100, 316, 1000, 2500, and 5000 µg/plate

using *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* WP2 uvrA, both with and without S9 metabolic activation, and incubated for 48 hours. The negative control was water alone; the positive controls were sodium azide, 4-nitro-o-phenylene-diamine, methyl methane sulfonate, and 2-aminoanthracene. All tests, including controls, were performed in triplicate.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with PureLo® at any concentration level in either the presence or absence of metabolic activation. The results of the assay demonstrated that, under the experimental conditions tested, Luo Han fruit concentrate did not cause gene mutations by base-pair changes or frameshifts in the genome of the tester strains used and is thus considered to be non-mutagenic in this assay.

4.1.4.2. Mammalian Micronucleus Test

A mammalian micronucleus test of murine peripheral blood cells was carried out under OECD Principles of Good Laboratory Practice (GLP) and in compliance with OECD Guideline No. 474 and EPA Health Effects Test Guideline No. OPPTS 870.5395, "Mammalian Erythrocyte Micronucleus Test" (BSL Bioservice 2009b). After a pre-experiment range-finding study, a maximum tolerable dose (MTD) was set at 2000 mg PureLo® Luo Han fruit concentrate/kg bw. This was the highest dose in the main experiment, while the mid dose was 0.5 MTD (1000 mg/kg bw) and the low dose was 0.2 MTD (400 mg/kg bw). The test animals, 5 of each sex per dose group, were healthy young adult NMRI mice, age 7 to 13 weeks, housed 5 per cage with free access to Altromin 1324 maintenance chow and water. The animals received the test article once intraperitoneally, dissolved in 10 ml saline water/kg bw. Additional animals received a negative control of pure saline water or positive control of cyclophosphamide dissolved in saline water. All animals were examined and peripheral blood was sampled from the tail vein at 44 hours, and the mice in the highest dose group and the negative control were examined and peripheral blood drawn at 68 hours. A minimum of 10,000 immature erythrocytes per animal were scored for the incidence of micronucleated immature erythrocytes; additionally, the ratio between immature and mature erythrocytes was determined and expressed as relative PCE.

With regard to the incidence of micronucleated immature erythrocytes, the negative control and all test groups were within the range of the historical negative control data and did not differ significantly from each other, while the positive control group showed a significant increase in micronucleus frequency. Similarly, the relative PCEs of the negative control and all tested groups were within the range of the historical negative control data and did not differ significantly from one another while the relative PCE of the positive control animals was significantly decreased.

The investigators concluded that under the experimental conditions tested, PureLo® Luo Han fruit concentrate did not induce structural or numerical chromosomal damage in the immature erythrocytes of the mouse, and is therefore considered to be non-mutagenic with respect to clastogenicity or aneugenicity in the mammalian erythrocyte micronucleus test.

4.2. Other Animal Studies of Luo Han Fruit Juice Extracts

A number of animal studies of Luo Han have been conducted with endpoints that are primarily nutritional rather than toxicological, and have generally been intended to assess potential benefits resulting from ingestion of Luo Han extracts. The absence of reported adverse effects in studies of such benefits, some of which involve as much as 13 weeks of feeding, corroborates the safety of Luo Han products.

Like many other fruit juices, Luo Han extracts appear to have antioxidant properties[1]. An *in vitro* study of low-density lipoprotein (LDL) oxidation (Takeo et al. 2002) provided evidence of an inhibitory effect of water-soluble mogrosides extracted from Luo Han fruit. The formation of conjugated dienes during copper-mediated LDL oxidation and of lipid peroxides during cell-mediated LDL oxidation were monitored in the presence or absence of water extract of Luo Han and the cucurbitane glycosides mogroside IV, mogroside V, 11-oxo-mogroside V, and siamenoside I.

Human plasma was prepared from fasting healthy men and LDL was isolated for incubation with the test compounds and exposed to copper sulfate; formation of conjugated dienes was monitored as a measure of oxidation. To test effects of Luo Han extract on cell-mediated LDL oxidation, human umbilical vein endothelial cells were cultured in the presence or absence of the test articles, and agarose gel electrophoresis and lipid peroxide assay were used to assess oxidation.

Statistically significant inhibition of LDL oxidation by Luo Han mogrosides was shown for both types of LDL oxidation (Takeo et al. 2002). Specifically, little activity was found due to mogroside V, mogroside IV, or siamenoside I; nearly all antioxidant activity was due to the presence of 11-oxo-mogroside V. The authors hypothesized that the *in vitro* inhibitory effect on LDL oxidation may be related to the free-radical scavenging capacity of the compounds. However, they noted that the physiological and pharmacological concentrations of Luo Han extract or individual mogrosides in plasma has not been defined—it has not been determined whether these compounds are substantially absorbed from human intestines—and thus the mechanism of inhibition remains to be elucidated.

Chen et al. (2007) also investigated the *in vitro* antioxidant activity of mogroside V and 11-oxo-mogroside V water-extracted from Luo Han fruits using a chemi-luminescence-based approach. The reactive oxygen species O_2^- was generated from a pyrogallol autooxidation system including the test mogrosides, and luminescence was counted every 3 seconds. Similarly, $^{\cdot}OH$ was generated in the presence of the mogrosides and luminescence was again counted every 3 seconds; luminescence was counted every 2 seconds in the action of mogrosides on H_2O_2. Finally, an assay of inhibitory action on DNA damage was conducted with luminescence counted every 10 seconds. Mogroside V showed greater inhibitory activity than did 11-oxo-mogroside V against the hydroxyl

[1] The component or components of Luo Han responsible for its antioxidant properties have not been elucidated. One likely contributor is the melanoidin content of dried fruits; these chemically complex Maillard polymers appear to be present at high concentrations and have been shown to have significant antioxidative properties (Borelli et al. 2002; Delgado-Andrade and Morales 2005; Delgado-Andrade et al. 2005; Daglia et al. 2008).

radical reactive oxygen species In the case of O_2^- and H_2O_2, however, mogroside V showed little inhibitory effect while 11-oxo-mogroside V showed a strong effect. 11-oxo-mogroside V also showed strong inhibitory effectiveness against induced DNA damage.

Antioxidant activity was also demonstrated *in vivo* by Yamada and Ogata (2001). Rats (strain, number, age, weight, and sex not reported) were placed on vitamin E-free diets; half were given Rakanka extract (dose and further description of the extract were not reported; Rakanka is the Japanese name for Luo Han products). While the hemolysis rate among control rats was 100% after 4 weeks, that in rats receiving Rakanka extract was less than 80%. Rats receiving Rakanka also showed significantly lower levels of serum cholesterol and serum lipid peroxide, indicating an antioxidant effect.

Antioxidant activity was cited by Hossen et al. (2005) as a likely mechanism for the significant effect of hot-water-extracted Luo Han extracts on nasal rubbing and scratching behavior in female ICR mice. Histamine was instilled into the bilateral nasal cavities of 6-10-week-old mice (number and caging arrangements not reported) that had received Luo Han glycoside extract (31% mogroside V) by gavage at daily doses of 0, 300 mg/kg, or 1000 mg/kg for 4 weeks. A dose-dependent inhibition of nasal rubbing was observed. A similar dose-dependent effect was found from the same doses of Luo Han extract administered to mice for 4 weeks prior to treatment with compound 48/80, which induced scratching behavior. No adverse effects due to the Luo Han extract were reported. Hossen et al. (2005) suggested that "the inhibitory effects of Lo Han Kuo on nasal rubbing and scratching behavior may be due to an inhibition of histamine release from mast cells through the prevention of superoxide anion generation."

It is likely that antioxidation also played a role in three findings (Ukiya et al. 2002; Konoshima and Takasaki 2002; Takasaki et al. 2003) of a possible inhibitory effect of ethanol-soluble glycosides extracted from Luo Han Kuo on tumor formation. In the former study, triterpene benzoates and glucosides isolated from an ethanol extract of Luo Han fruit juice showed significant *in vitro* inhibitory effects on the induction of Epstein-Barr virus early antigen (EBV-EA) by 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (Epstein-Barr virus genome-carrying human lymphoblastoid cells). However, water-soluble fractions showed considerably weaker inhibitory properties than did the less polar n-hexane-soluble fraction.

Konoshima and Takasaki (2002) tested the cancer-chemopreventive effects of several cucurbitane glycosides: carnosifloside I, V, and VI isolated from *Hemsleya carnosiflora*, scandenoside R2, R4, and R6 from *Hemsleya panacis-scandens*, and mogroside V from *S. grosvenorii*. The methods of isolation were not reported, although another study from the same laboratory (Takasaki et al. 2003) reported the use of ethanol to perform Luo Han extraction.

Like Ukiya et al. (2002), Konoshima and Takasaki (2002) carried out a short-term synergistic assay on EBV-EA induction with TPA, finding the strongest degree of inhibition by scandenoside R6, followed by carnosifloside VI and mogroside V. All of the glycosides tested showed significant degrees of inhibition of EBV-EA. This *in vitro* test was followed by a two-stage mouse skin carcinogenesis test using mogroside V alone. The backs of ICR mice (sex, age, and number not reported) were shaved and

topically treated with 7,12-dimethylbenz[a] anthracene (DMBA) in acetone as an initiator, and topically treated 1 week later with TPA as a promoter. Topical treatment with mogroside V significantly reduced the incidence of papillomas within 9 weeks, apparently delaying papilloma formation.

In the third study (Takasaki et al. 2003), these results were replicated, again using Raji cells cultured in fetal bovine serum as indicators of EBV-EA. Additionally, two *in vivo* studies were performed using 6-week-old pathogen-free female ICR and SENCAR mice. In the first study, two-stage mouse skin carcinogenesis model employed by Konoshima and Takasaki (2002) was used: the backs of 30 ICR mice were shaved and topically treated with DMBA as an initiator, and topically treated 1 week later with TPA as a promoter. Half of the mice (n = 15) were topically treated with 11-oxo-mogroside V 1 hour before the promotion treatment; the incidence of papillomas was observed for 20 weeks. In the second study, 45 SENCAR mice were shaved a topically treated with peroxynitrite as an initiator, and promoted by application of TPA 1 week later. One group (n=15) received mogroside V in their drinking water (2.5 mg/100 ml) from 1 week before to 1 week after the initiation treatment, while another group received the same dose of 11-oxo-mogroside V; the third group was used as a control. Again the incidence of papillomas was observed over 20 weeks.

The *in vitro* tests found some degree of inhibition of EBV-EA by all four mogrosides tested, although 11-oxo-mogroside V had greater effect than mogroside V, mogroside IV, or siamenoside I (Takasaki et al. 2003). In the first *in vivo* test, with DMBA as the initiator, topical treatment with 11-oxo-mogroside V showed significant inhibition of papilloma development. In the second *in vivo* study, both 11-oxo-mogroside V and mogroside V, ingested in the drinking water, were found to have a significant inhibitory effect on mouse skin tumors induced by peroxynitrite. The doses of mogroside in the drinking water had no effect on the body weight of the mice.

In a similar vein, Akihisa et al. (2007) found that 8 ethanol-soluble mogrosides exerted significant inhibitory effect on EBV-EA activation induced by TPA *in vitro*, but only weak inhibitory effects on the activation of (±)-(E)-methyl-2-[(E0-hydroxyimino]-5-nitro-6-methoxy-3-hexemide, a nitric oxide donor, in Chang liver cells. However, the tested mogrosides are insoluble or only slightly soluble in water (Ukiya et al. 2002) and thus are most likely present at most in trace quantities in PureLo®.

The anti-cariogenic effects of a maceration extract of Luo Han Kuo (not further described, but apparently a water extract), sucrose, glucose, fructose, and beet sugar were compared by exploring their effects on *Streptococcus mutans* (Mu 1998). Growth, glass-rod adherence, and acid production were observed. All three endpoints were significantly lower with the Luo Han extract than with the other sweeteners.

The abilities of five mogrosides to inhibit the activity of mammal DNA polymerases and suppress the growth of human cancer cells were studied *in vitro* by Mizushina et al. (2006) using polymerase extracted from calf thymus and a human cancer cell line derived from a cancer patient. The water-soluble mogrosides found in PureLo® (primarily mogroside V) did not influence the inhibition of DAN polymerase activity or human cancer cell proliferation, while the methanol-soluble mogroside I E_1 had

significant inhibitory effect. This result again highlights the importance of the method of extraction used in performing research with Luo Han extracts.

Suzuki et al. (2005) studied the effect of both crude hot-water extracts from Luo Han fruit and purified mogroside on the postprandial rise in blood glucose level of rats given either glucose or maltose a few minutes later. Analysis of the crude extract showed content of 2.1% mogroside V, 0.8% mogroside IV, 0.7% mogroside III, and 0.3% siamenoside I; the corresponding percentages in the purified extract were 30.9%, 1.6%, 0.8%, and 1.4%. Thus, the primary effect of the purification procedure was to increase the concentration of mogroside V.

Six-week-old male Wistar rats (number not reported) were fasted and then given 100 mg/kg bw of crude extract, purified mogrosides, or water by gavage. Three minutes later, 2000 mg/kg bw of either maltose or glucose solution was also administered by gavage. Blood samples were collected from the tail vein prior to the first administration and 30, 60, 90, and 120 minutes after the second administration and analyzed for glucose level. In a separate *in vitro* test, sucrose or maltose was added to a rat intestinal enzyme solution along with different amounts of crude or purified Luo Han extract or individual mogrosides; inhibition of sucrase and maltase activity was measured.

No inhibitory effect was found for either crude or purified extract on blood glucose levels when glucose was administered, but significant inhibition was found after administration of maltose, suggesting that Luo Han may exert a small antihyperglycemic effect by inhibiting maltase in the small intestinal epithelium. Inhibition of maltase was confirmed in the *in vitro* studies for both the crude and purified extract, as well as for mogroside V, mogroside IV, and siamenoside I, but only slightly for mogroside III. Little effect was found on sucrase activity. Suzuki et al. (2005) compared this finding with the inhibition of α-glucosidase reported for stevioside and the inhibition of sucrose transported reported for glycyrrhizin; it is also similar to the recognized sucrase inhibitory effect of dietary phenolic compounds (Welsch et al. 1989). The authors suggested that, if *in vivo* research confirms the results of the *in vitro* experiment, this inhibitory effect would be beneficial in reducing the postprandial blood glucose response. However, as Ohta et al. (2002) emphasized, "*in vitro* inhibitory activity is not always related to the *in vivo* activity" of enzyme inhibition.

In assessing the safety implications of the *in vitro* evidence for maltase inhibition reported by Susuki et al. (2005), the research on inhibition of sucrase by natural dietary substances reported by Preuss et al. (2007) is relevant. After confirming the inhibitory effects of bean and hibiscus extracts and L-arabinose, the authors concluded first that such substances exert their effect by affecting absorption rather than overall metabolism, and second that at reasonable doses these substances are safe and possibly beneficial.

A study of antidiabetic effects of Luo Han extracts was conducted by Suzuki et al. (2007), using spontaneously diabetic Goto-Kakizaki (GK) rats. The GK rat was selectively bred from a starting colony of Wistar rats; it exhibits impaired insulin secretion, insulin resistance, and abnormal glucose metabolism, but do not become obese and are not hyperlipidemic. While this was not primarily designed as a toxicity study, it included a 13-week feeding regimen and a number of safety-related endpoints. The test

article was Luo Han fruits crushed and boiled in water with the water-soluble fraction concentrated to a paste containing 2.1% mogroside V.

Male 5-week-old GK rats were housed individually and acclimated to the control diet for 2 weeks and then randomized to a control group and a test group (n = 10 rats/ group) that received the control diet supplemented with 0.4% Luo Han extract. Feed and water were available *ad libitum*; feed intake and body weight were measured every other day and blood was collected from the tail vein biweekly. An oral glucose tolerance test was performed at week 7 of treatment: 1 g glucose/kg bw was intubated orally and tail-vein blood was collected at 0, 30, 60, 90, and 120 minutes. During weeks 11 and 12, rats were individually placed in metabolism cages for 3 days before urine was collected for 2 days.

At the end of the 13-week feeding period (age 20 weeks), rats were fasted for 16 hours prior to sacrifice, blood was collected from the vena cava, and the heart, liver, kidney, spleen, pancreas, and small intestine were collected and weighed. Pancreatic insulin levels were determined and thiobarbituric acid-reactive substance (TBARS) levels of the liver, kidney, pancreas, and plasma were determined. Blood measures included triacylglycerol, total cholesterol, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, γ-glutamyl transpeptidase, lactic dehydrogenase, and alkaline phosphatase.

The average daily feed intake was about 15 g and did not differ between groups, nor did body weights or organ weights (Suzuki et al. 2007). Luo Han extract significantly improved the insulin response and reduced the plasma glucose level in the glucose tolerance test. Pancreatic insulin levels were significantly higher and TBARS were lower in the rats that received Luo Han, suggesting an antioxidative effect on lipid peroxidation. Urine volume and urinary albumin were significantly reduced, suggesting the attenuation of diabetes-induced kidney damage. Additionally, glutamic oxaloacetic transaminase and γ-glutamyl transpeptidase were significantly lower and glutamic pyruvic transaminase, lactic dehydrogenase, and alkaline phosphatase were non-significantly lower in the Luo Han group, suggesting that the decline of liver function caused by diabetes is attenuated by long-term supplementation with Luo Han extract. The authors reported that "13-week supplementation of [Luo Han extract] did not show any adverse effects in GK rats, including feeding behaviour, body weight and various biochemical parameters in various organs."

Lin et al. (2007) studied the effects of a hot-water Luo Han extract on diabetic rabbits, using male New Zealand white rabbits fed a high fat/high sucrose diet. Thirty rabbits were randomly assigned to 5 groups (n = 6 rabbits/group): normal control receiving regular rabbit chow for 8 weeks; diabetic control receiving chow with added 10% lard and 37% sucrose for 8 weeks; and diabetic experimental groups receiving the high fat/high sucrose chow for 4 weeks before also receiving 50, 100, or 200 mg Luo Han extract/kg bw/day for the remaining 4 weeks. Animals were fed 35 g chow/kg bw/day each morning and given free access to water. Food consumption was measured daily. At the end of the feeding, blood was taken from auricular veins after overnight fast and analyzed for glucose, insulin, total cholesterol, HDL cholesterol, and triacylglycerol.

The highest fasting levels of glucose and insulin were seen in the diabetic control animals. Addition of Luo Han extract had no effect on insulin level, but significantly reduced glucose nearly to the level of the normal control group, although no dose-response relationship was apparent. The mid- and high-dose Luo Han treatments also significantly reduced the high total cholesterol and triacylglycerol levels of the diabetic control animals, but not to the level of the normal controls. Similarly, mid- and high-dose levels of Luo Han raised HDL cholesterol, but again not all the way to the normal controls. The low-dose Luo Han group was not significantly different from the diabetic controls, but no dose-dependent effect was seen between the mid- and high-dose groups. Lin et al. (2007) concluded that the Luo Han extract not only ameliorated the lipid disorder, but also lowered plasma glucose levels. No adverse effects were reported for any dose of Luo Han extract.

Yasuno et al. (2008) studied the ability of Luo Han extract to ameliorate the hepatocarcinogenic effect of α-[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene (PBO), an insecticide believed to exert its hepatocarcinogenic effect through the generation of reactive oxygen species. Male F344/N Slc rats aged 4 weeks and weighting an average of 53.75 g were randomly assigned to one of 3 groups (n = 12 rats/group, caged in groups of 4): control, PBO-treated, or PBO + Luo Han extract-treated. The Luo Han extract tested in this study was provided by Saraya Co., Ltd., of Japan; although it was not characterized in this article it is most likely the same extract as was provided by Saraya for the study by Jin et al. (2007), discussed earlier, a water extract nearly identical with PureLo®, containing 31.4% mogroside V. The extract was added to the water, which as available *ad libitum*, at a concentration of 1000 ppm.

Animals consumed their assigned diets and water for 2 weeks, and then animals in all 3 groups were given a single intraperitoneal injection of N-diethylnitrosamine to initiate hepatocarcinogenesis and subjected to two-thirds partial hepatectomy 1 week later. Test animals had 2% PBO added to their feed, while the Luo Han group continued to receive water containing 1000 ppm extract. Feeding continued for 7 weeks. Feed consumption and body weight were measured weekly. After sacrifice, livers were excised, weighed, and fixed for histopathological examination. Liver microsomes were obtained from 3 rats from each group for measurement of production of reactive oxygen species, while lipid peroxidation in the livers was assessed by measurement thiobarbituric acid-reactive substances (TBARS) in other rats from each group. Glutathione S-transferase and glutathione peroxidase activities were measured.

Seven rats died as a result of the hepatectomy, leaving 8 rats in the control group, 10 in the PBO group, and 11 in the PBO + extract group. Body weights were significantly decreased and both absolute and relative liver weights were significantly increased in both PBO groups, which did not differ from each other. Histopathology revealed centrilobular hepatocytic hypertrophy in the PBO groups; again, the ingestion of Luo Han extract had no effect. Production of reactive oxygen species was significantly increased in the PBO groups and was unaffected by Luo Han extract. TBARS, on the other hand, which were significantly elevated in both PBO groups, were significantly lower in the extract group. Hepatic glutathione S-transferase activity was significantly raised in the PBO groups, and significantly further raised in the group receiving Luo Han extract, while glutathione peroxidase activity was significantly elevated only in the group

receiving both PBO and extract. The authors noted that Luo Han extract inhibited lipid peroxidation with no evident adverse effects.

A generally similar study again used the Saraya Luo Han extract (Matsumoto et al. 2009), this time providing a description of the article as a water extract with approximately 31% mogroside V. The objective of the study was to assess the suppressive effect of Luo Han extract on the promotion of hepatocellular proliferative lesions by 4,6-diamino-2-cyclopropylaminopyrimidine-5-carbonitrile (DC), a known non-genotoxic hepatocarcinogen that may act through production of reactive oxygen species.

Two experiments were performed. In the first, 5-week-old male ICR mice were acclimated for 1 week before being assigned to 3 groups (n = 8/group): control, DC, and DC + Luo Han extract. The extract was administered in the drinking water at a concentration of 2500 ppm, beginning 1 week prior to further treatment. All mice were subjected to two-thirds partial hepatectomy and given intraperitoneal injections of diethylnitrosamine to induce hepatocarcinogenesis. One week later, DC was added to the diets of the 2 test groups and feeding continued for 9 weeks. At the end of feeding, the mice were weighed, sacrificed, and their livers excised, weighed, and prepared for histological and histochemical examination.

Three mice died due to the hepatectomy (Matsumoto et al. 2009). Body weights did not differ across the 3 groups. Relative liver weights were significantly increased in both DC groups, which did not differ from each other. Centrilobular hepatocytic hypertrophy was observed in both DC groups; again, the ingestion of Luo Han extract had no effect. The number of γ-glutamyl-transpeptidase-positive hepatocytes was significantly elevated in both DC groups, but was significantly lower in the extract group. TBARS, similarly, were significantly elevated in both DC groups but significantly lower in the Luo Han group.

In the second experiment by Matsumoto et al. (2009), 20 male C57BL/6J and DBA/2J mice were divided into 6 groups, each with 3-4 mice. Two groups were controls and the other 4 groups received DC in their feed; 2 of these groups also received water containing 2500 ppm Luo Han extract. Feeding continued for 3 weeks, after which liver samples were taken and gene expression of *Cyp1a1* was measured by reverse transcription polymerase chain reaction (RT-PCR). Both strains of mice showed significantly elevated gene expression due to DC exposure; this level was reduced by Luo Han extract in C57BL/6J mice but not in DBA/2J mice.

Based on the findings of the two experiments, Matsumoto et al. (2009) concluded that Luo Han extract may suppress DC-induced generation of reactive oxygen species.

4.3. Fermentation of Luo Han Extract By Colonic Microbiota

A complex resident gastrointestinal microbiota is present in humans. While the transit of residual foods through the stomach and small intestine is probably too rapid for the microbiome to exert a significant impact, this slows markedly in the colon. As a result, colonic microorganisms have ample opportunity to degrade available substrates

through the anaerobic metabolic process known as fermentation. Fermentation by gut bacteria consists of a series of energy yielding reactions that do not use oxygen in the respiratory chains. The electron acceptors may be organic (e.g., some products of the fermentation) or inorganic (e.g., sulfate, nitrate). As carbohydrates form the principal precursors for fermentation, ATP is usually formed through substrate level phosphorylation by saccharolytic microorganisms.

The fermentation of PureLo® Luo Han fruit concentrate by human gut bacteria was assessed in a continuous culture system (Gibson 2007). The model system at Reading University was validated against gut contents from sudden-death victims and gives a close analogy to bacterial activities and composition in different areas of the hindgut. The gut model system consists of three vessels (V_1, V_2 and V_3) aligned in series and maintained under anaerobic conditions at 37°C with pH in the three vessels maintained at 5.5, 6.2 and 6.8, respectively. V_1 replicates the proximal colon with a more acidic pH, high substrate availability, and more rapid transit of contents, while V_3 resembles the distal colon with a neutral pH, limited substrate availability, and slow flow rate. A peristaltic pump moves contents through the three vessels. Fecal samples were obtained from 3 healthy individuals and mixed to form a slurry; 100 ml of the slurry was added to each vessel of the gut model, which was run in triplicate.

At each sampling time point, 3 ml was removed from each vessel for enumeration of different bacterial groups using fluorescent *in situ* hybridization (FISH). This was used for the enumeration of total bacterial load as well as four predominant gut bacterial genera: bifidobacteria, bacteroides, lactobacilli, and clostridia. Any fermentation of PureLo® would be reflected in a 0.5 log or higher increase the microbial numbers.

Only minor fluctuations were seen in the enumerations, both of total bacteria and of the four marker genera. Based on this research therefore, Gibson (2007) concluded that Luo Han fruit concentrate is apparently not metabolized by the colonic microbiota.

Yang et al. (2007), in a Chinese article for which only an abstract was available in English, reported isolating mogroside III (which is found in only trace levels in water extracts of Luo Han) and incubating the glycoside with crude enzymes of human intestinal bacteria under anaerobic conditions at 37°C. Successive deglycosylation at C-3 of the glucosyl group and C-24 of the gentiobiosyl group resulted in biotransformation to mogroside II-A_1 and mogrol (the aglyconic form). Since the predominant mogroside in PureLo® is mogroside V rather than mogroside III, this result does not conflict with the Gibson (2007) findings, but it is unclear whether the difference was due to the mogroside or to the methodologies, which may have involved unrealistically high levels of enzyme activity in the Yang et al. (2007) research.

4.4. Cytotoxicity and Anti-Inflammatory Activity of Luo Han Extracts

Li et al. (2007a) tested the cytotoxic activity of 12 cucurbitane triterpene glycosides and flavonol glycosides isolated (mostly by methanol extraction) from the ripe or unripe fruits of Luo Han against HCT-116 colon cancer cells and SMMC-7721 hepatoma cells. Concentrations of 50, 100, 200, and 400 μg/ml were tested of each of the following glycosides:

11-oxo-mogroside I A_1	11-dehydroxymogroside III
20-hydroxy-11-oxo-mogroside I A_2	kaempferol 3,7-α-L-dirhamnopyranoside
mogroside II E	mogroside IV
11-oxo-mogroside II E	11-oxo-mogroside IV
mogroside III	mogroside V
11-oxo-mogroside III	kaempferol 7-α-L-rhamnopyranoside

None of these compounds exhibited any cytotoxic activities against cultured tumor cell lines.

In order to assess whether PureLo® Luo Han fruit concentrate possesses any anti-inflammatory activity, the effect of Luo Han fruit concentrate on the *in vitro* production of tumor necrosis factor α (TNFα) from a mouse macrophage cell line was determined (Skinner & Adaim 2006). As TNFα is a major inflammatory mediator, a reduction in the production of TNFα would indicate that Luo Han fruit concentrate may have anti-inflammatory activity. Prior to this, the effect of Luo Han fruit concentrate on the viability of the cells was determined so that cytotoxicity could be eliminated as a potential cause of any measured effects on the cells.

PureLo® Luo Han fruit concentrate and two controls, glucose and indomethacin (a non-steroidal anti-inflammatory compound), were tested for cytotoxicity on RAW 264.7 cells (mouse macrophage cell line ATTC Cat No.TIB-71) using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Skinner & Adaim 2006). This assay measures the integrity of mitochondria within living cells. They were measured at doses ranging from 0.04 to 10,000 μg/ml for Luo Han fruit concentrate and glucose and from 5 to 1000 μM for indomethacin. Luo Han fruit concentrate, glucose, and indomethacin were then tested for their ability to inhibit TNFα production from cultured mouse macrophage RAW 264.7 cells. As indomethacin was cytotoxic at the higher doses, anti-inflammatory activity was only measured at doses ranging from 5-100 μM. PureLo® and glucose were tested over the whole dose range, although Luo Han fruit concentrate was slightly toxic to the cells at doses above 1,000 μg/ml.

The macrophages were plated out at 2×10^5 cells/ml in 96 well plates for 18 hours and doses of Luo Han fruit concentrate, glucose, and indomethacin were added to cells and incubated for a further 18 hours. Also included were untreated cells in media alone. The cells were then stimulated with lipopolysaccharide (LPS) at a final concentration of 500 ng/ml for 6 hours. Supernatants were collected and TNFα was assayed.

Untreated cells cultured with media alone produced 91±16 pg/ml TNFα; with LPS stimulation 1098±35 pg/ml TNFα was produced. When cells were treated with Luo Han fruit concentrate prior to LPS there was no reduction in the production of TNFα at any dose tested. Glucose also had no effect and, as expected, indomethacin reduced TNFα production in a dose-dependent manner. The investigators concluded that, as measured by the *in vitro* inhibition of TNFα from a mouse macrophage cell line, Luo Han fruit concentrate did not exhibit anti-inflammatory activity at doses as high as 10 mg/ml (Skinner & Adaim 2006).

4.5. Human Studies of Luo Han Extracts

In a cross-over design, Xu et al. (2005a) assessed the comparative effect of consumption of PureLo® Luo Han fruit concentrate and sucrose on blood glucose level. After fasting overnight, 5 healthy men and 5 healthy women aged 19-25 years consumed 200 mg/kg bw of Luo Han fruit concentrate dissolved in water. Their blood glucose levels were tested at 0, 15, 30, 60, 120, and 180 minutes after dosing. Three days later, the same 10 participants consumed 3000 mg/kg bw of sucrose dissolved in water, again after an overnight fast, and blood samples were taken at the same time intervals. While ingestion of sucrose resulted in a 70% increase in blood glucose level during the first 15 minutes, gradually decreasing to the starting level over 3 hours, ingestion of Luo Han fruit concentrate had no effect on blood glucose. These results are exhibited in Table 8.

Table 8. Effects of Luo Han Fruit Concentrate and Sucrose on Blood Glucose Level.

Time After Dosing	Blood Glucose Level (mmol/L; mean±S.D.)	
	PureLo® (200 mg/kg bw)	Sucrose (3000 mg/kg bw)
0 minutes	4.59±0.45	4.52±0.44
15 minutes	4.50±0.44	7.68±0.74
30 minutes	4.76±0.33	6.97±0.91
60 minutes	4.70±0.26	6.00±1.35
120 minutes	4.46±0.34	5.09±1.07
180 minutes	4.56±0.51	4.42±0.95
Source: Xu et al., 2005a		

Xu et al. (2005b) used a similar cross-over design to assess the effect of PureLo® Luo Han fruit concentrate and that of water on blood levels of liver enzymes. Six healthy males aged 19-25 years fasted overnight and then consumed 200 mg/kg bw of Luo Han fruit concentrate dissolved in water; 3 days later they consumed only water. On both days, blood samples were taken at 0, 1, 2, 3, and 6 hours after administration. Five liver enzymes were analyzed: alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). There was no statistically significant change in the blood level of any of these enzymes over time, nor any difference between enzyme levels after dosing with Luo Han fruit concentrate or with water (Table 9).

Table 9. Effects of Luo Han Fruit Concentrate on Clinical Chemistries.

	Liver Enzyme (mmol/L; mean±S.D.)				
Time and Condition	ALP	GGT	ALT	AST	LDH
Hour 0					
PureLo®	77.49±23.30	13.75±5.02	10.60±4.65	20.03±5.07	170.60±24.17
Water	53.15±11.72	10.65±3.14	9.72±5.18	14.67±2.41	106.50±27.45
Hour 1					
PureLo®	72.65±17.88	11.91±3.32	10.03±7.33	19.95±4.32	180.36±51.83
Water	37.50±19.85	7.37±4.64	7.90±4.17	10.90±5.65	75.01±39.33
Hour 2					
PureLo®	68.76±17.88	10.97±3.43	8.78±3.52	17.13±3.26	176.03±22.81
Water	49.80±12.09	9.58±3.50	7.83±4.56	13.00±1.74	93.04±28.56
Hour 3					
PureLo®	70.03±22.89	10.57±3.20	8.82±3.23	17.182.50	160.67±32.24
Water	47.85±10.01	9.59±3.27	8.70±2.82	14.02±2.20	91.47±16.49
Hour 6					
PureLo®	75.74±15.36	12.78±3.90	10.28±5.24	20.53±4.40	175.60±50.23
Water	50.11±11.37	9.42±3.04	10.18±5.60	15.35±2.53	114.27±39.90

ALP = alkaline phosphatase
GGT = γ-glutamyl transpeptidase
ALT = alanine aminotransferase
AST = aspartate aminotransferase
LDH = lactate dehydrogenase

Source: Xu et al., 2005b

4.6. Ribosome Inactivating Proteins

Some members of the genus *Momorica*, which was at one time regarded as including Luo Han, which is now classified in genus *Siraitia*, have been reported to contain ribosome inactivating proteins (RIP) in their seeds. Most specifically, the presence of these proteins has been demonstrated in the seeds of *Momorica charantia*, a cucurbitane distinctly different from *Siraitia grosvenorii*. An ethanol extract of *M. charantia* was orally administered to 9 dogs (strain not reported) for 20, 40, or 60 days at a dose of 1750 mg/day (125 mg/kg bw/day) while 3 dogs served as controls (Dixit et al. 1978). Body weights were unaffected and few changes were noted in serum clinical chemistries or hematology, but numerous testicular biochemical and histological effects were seen. Antispermatogenic effects were observed as early as 20 days, became more prominent by day 40, and led to mass atrophy of the spermatogenic elements by day 60. By day 60, significantly decreased protein, RNA, and sialic acid contents of the testes were noted. The authors of this study did not report information on the degree of concentration of *M. charantia* that occurred in preparing the "thick syrup" used as the test

article, so it is not possible to determine how much *M. charantia* gourd was needed to prepare the test article. Additionally, the specific component of the extract responsible for the antispermatogenic effect was not identified.

Tsang and Ng (2001) identified the RIP in *M. charantia* seeds as α- and β-momorcharins. In an analysis of *S. grosvenorii* seeds, these authors identified a different RIP, which they termed momorgrosvin, a single-chained glycoprotein with a molecular weight of 27.7 kDa. (It should be noted that the isolation procedure was based on acetone precipitation. Seeds of *S. grosvenorii* are not normally included in the manufacture of PureLo® Luo Han fruit concentrate, and acetone is not used in the extraction process.) Tsang and Ng (2001) reported that 25 g of powdered decoated seeds were required to obtain 60 μg momorgrosvin, a yield of 0.00024%, and observed that this was nearly 3 orders of magnitude less than the yield of momorcharins from *M. charantia* seeds, 48 mg/25 g seeds, or 0.192%. Further, the authors reported that only a 39% homology exists between momorcharins from *M. charantia* and momorgrosvin from *S. grosvenorii.*

In addition to their presence in cucurbitans, RIP are widely distributed in the plant kingdom, occurring in the seeds of such important food crops as wheat, rye, and barley. Stirpe and Barbieri (1986) reported that, "A survey revealed the presence of proteins with the characteristics of type 1 RIPs [single-chain proteins such as momorgrosvin] in most plant materials examined, including seeds, roots, leaves and latices, thus confirming the wide distribution of RIPs." They also noted that concentrations range from less than 1 to over 100 mg/100 g. (Note that the concentration of momorgrosvin in Luo Han seed—which is far greater than its concentration in the fruit as a whole—was reported by Tsang and Ng [2001] as only 0.24 mg/100 g.)

In a review of RIP, Roberts and Selitrennikoff (1986) concluded that, as opposed to two-chain (type 2) RIP such as ricin, abrin, and modeccin, the type 1 group:

> "... consists of proteins that are single polypeptide chains and which resemble the A-chains of plant toxins in the physical-chemical and enzymic properties, as well as in their amino-terminal amino acid sequences. These proteins are relatively non-toxic, for they lack a B-chain and bind poorly to mammalian cells" (p 19).

Of particular importance is that RIP are quickly deactivated by heating. Coleman and Roberts (1982) demonstrated that RIP heated for 15 minutes at 90°C lost over 90% of their biological activity. Since PureLo® Luo Han fruit concentrate is decocted in boiling water for 1 hour, it is unlikely that any RIP present would retain significant activity.

Type 1 RIP occur at relatively high concentrations in a large variety of widely consumed foods with no indication of adverse effects. They are present in the seeds of *S. grosvenorii* at much smaller concentrations, and because their activity would almost certainly be completely destroyed by the extraction process, it is concluded that no safety concern is raised regarding the intended use of Luo Han fruit concentrate.

4.7. History of Use of Luo Han Guo

Luo Han has been widely consumed as a decoction of the dried fruit, primarily in its native China but also in the U.S., where the dried fruit is imported and sold most frequently in Chinese food stores. Additionally, extracts of the Luo Han fruit have also been the source of numerous beverages, sweeteners, and other products that are also widely used in China, Japan, Australia and New Zealand, Europe, and the U.S.

While PureLo® is a condensed fruit extract, its mogroside concentration is no greater than is found in the traditional decoction, and the potential intake of mogrosides from PureLo® Luo Han fruit concentrate, based on its intended uses, is no higher than that which occurs through consumption of the traditional decoction. As discussed in the previous chapter (see Table 7), an extremely conservative estimate of the 90th percentile potential daily intake of Luo Han fruit concentrate is 6.8 mg/kg bw/day, or 400 mg/day for a 60-kg individual. Since the product contains about 30% mogroside V, this level of intake would result in mogroside V intake of about 120 mg. The dried Luo Han fruit is about 0.5% or more mogroside V, and thus 120 mg of mogroside V is derived from about 24 g of the fruit (about half a medium sized fresh fruit) or a lower amount of traditional fruit concentrate, well within a likely range of daily consumption from traditional uses of Luo Han decoctions.

4.7.1. Use of the Fruit

4.7.1.1. Traditional Chinese Uses

The Chinese plant Luo Han Guo (*Siraitia grosvenorii*) is a perennial vine in the Cucurbitaceae (cucumber or melon) family. Luo Han fruits ("guo" or "kuo" means fruit) are used both inside and outside the People's Republic of China as foods, beverages, seasonings, and traditional medicines. In a survey of sweeteners isolated from plant sources, Kinghorn and Soejarto (2002) reported that this fruit is widely used as a dietary and medicinal food throughout China and Southeast Asia.

Historically Luo Han Guo grew wild throughout the mountainous terrain of Southwest China. Historic Chinese writings reference Song Dynasty monks brewing Luo Han for medicinal beverages over 800 years ago. By 1800, Luo Han Guo was a broadly cultivated crop in the region. According to the *Official Historical Records of Guangxi Province*, Luo Han Guo has been cultivated and the fruit harvested for human use since the start of record keeping over 300 years ago. The *Guangxi History of Chinese Medicine*, printed in 1963, provides a detailed record of harvested material being used as a medicine in 1885 in Guangxi Province. According to the *Guangxi History of Chinese Medicine*, "Luo Han Guo is sweet, not toxic, and beneficial to lung and spleen ... It can stop coughing, improve digestion, and serve as a refrigerant. It can be used to cure coughing, constipation, etc." It is important to note that the bases for the attribution of these benefits are cultural and anecdotal; there are no published data to support them, although the high free sugar content of crude Luo Han extracts makes them hygroscopic and likely helpful in constipation. It might also be noted that, according to Anderson and Anderson (1977), watercress and carrots are regarded in south China primarily as traditional "cooling" medicines rather than as foods.

There have in fact been instances where regulatory authorities have required health-benefit claims to be removed from marketing material for Luo Han products. For example, the *Star* newspaper reported on July 30, 2002, that Dragon River Health Products was ordered by the Singapore Health Sciences Authority to remove claims promoting its Luo Han Kuo Tea as useful in relieving respiratory ailments—one of Luo Han's principal folk-medical uses.

There is an 1813 written reference from the scholar Lui Dong-Ing attesting to Luo Han Guo's familiar presence as a cultivated crop. The usage of Luo Han fruits before 1958 was described in the Chinese "Certificate of Luo Han Guo Glucosides as a Food Additive." The related portions are translated here:

> *"Luo Han Guo has been used by Chinese for drink and medicine for more than 300 years. Its value as a natural sweetener and an herb medicine has been well recognized. Luo Han Guo is a unique herb only found in the south part of China, especially around Yongfu, Lingqua, Longshen areas at the north of Guangxi Province. As the production center of Luo Han Guo, the Guangxi Yongfu area produces approximately 70% of Luo Han Guo in China. According to the County History of Guangxi Yongfu, local people have cultivated Luo Han Guo crops and collected the fruits for more than three hundred years."*

The most complete report in English on the traditional uses of *Siraitia grosvenorii* in China is an unpublished manuscript written in 1938 by G.W. Groff and Hoh Hin Cheung. In China, the fruits were reported to be frequently used as the main ingredient in "cooling drinks" or "cooling tea." The juice of fresh fruits was reported to be very sweet. Groff and Hoh (1938) reported that all *Siraitia grosvenorii* fruits of commerce were carefully dried over fires in special drying sheds.

Groff and Hoh (1938) also reported that the "Luo Han fruit of commerce, when cooked with pork or steeped with tea, provides a common Chinese household remedy for colds and congestion of the lungs." Groff and Hoh concluded from interviews and the fact that *Siraitia grosvenorii* was not listed in several classical Chinese medical texts that the plant had only become extensively used in China in recent history. However, the development of distinct cultivars and the amount of knowledge of Luo Han's growth, pollination, climatic, and drying requirements implies a fairly long history of use by some group of people. The origin of the plant's common name is uncertain, but *Siraitia grosvenorii* in Chinese culture is associated with the saints that surrounded Buddha and "guo" or "kuo" generally refers to a fruit. If the plant was brought into cultivation by aboriginal or tribal people, as proposed by Groff and Hoh (1938), then the common name may have had a different meaning to the original tribe. Although Swingle (1941) reported the plant to be cultivated by the non-Chinese Miao-tze people, the Zhuang are the most numerous of the more than ten nationalities that live in Guangxi Zhuang autonomous region.

Dried Luo Han fruits are used whole, powdered, or in block forms as sources of beverages, seasonings, and traditional medicines as analgesics, expectorants, antitussives, or to treat infiltration of the lungs (Takamoto, et al. 1978). The *Encyclopedia of*

Traditional Chinese Medicine (Jiangsu New Medical College, 1977) recommended the use of dried fruits as good for lung complaints including dry coughs and as a laxative at a consumption rate of 10-15 g or one dried fruit boiled in water per day. The fruits are very sweet and are used in folk medicine for the treatment of sore throats and for stomach and intestinal disorders as well as colds (Makapugay et al. 1985). (These folk therapies may well be successful; Luo Han is high in vitamin C and the high fructose content of crude extracts would tend to make them viscous, which might result in a soothing effect due to prolonged contact with the throat.) The Chinese book *Fruit as Medicine* (Dai and Liu, 1986) reports the fruits are used for heat stroke with thirst, acute and chronic throat inflammation, aphonia, chronic cough, constipation in the aged, and as a sugar substitute for diabetics. In general, the preparation method is to boil or simmer the fruit in water and drink it as an herb tea. As a sugar substitute in cooking, the fruits may be simmered into thick syrup and added during the preparation of the food.

According to an article in the Journal of Chinese Medicine Information in 1996, the production of Luo Han Guo has increased significantly in the past twenty years. The annual production volume in 1995 was about 25-30 million kg. The authors of the article cited several reasons for this increase. Before 1970, Luo Han Guo had been used only in the Guangxi and Guangdong areas as a local Chinese herb medicine. It was seldom used in other areas. After the medical application of Luo Han Guo was recorded in the 1991 edition of the *Pharmacopoeia of People's Republic of China*, use spread to all of China. Additionally, the range of applications increased. Before the 1970s, Luo Han Guo had been used only as a traditional medicine. Since 1980, the medical usage of Luo Han Guo has been increased with the growth of the Chinese medicine industry. As of 1995 there were more than 20 over-the-counter Chinese medicine products and approximately 10 health products using Luo Han Guo as the primary constituent. In addition, Luo Han Guo is also widely used to prepare drinks. The authors noted that it is sweet, tasty, refrigerant, and believed to be helpful for the lung. Luo Han Guo drink is very popular and contributes to the sales of Luo Han Guo. Finally, the export volume increased. Luo Han Guo is a traditional export item, but since 1980 Luo Han fruit and its over-the-counter medicine products have entered the European and American markets. Exports of Luo Han Guo have been increasing about 2% per year, resulting in a six-fold increase from 5 million pieces in 1970 to 35 million pieces of Luo Han Guo fruit in 1995. Luo Han Guo is mainly exported from Guangzo, Shanghai, and Tianjin.

The longest consistent use of Luo Han Guo by a large population is found in Guangxi Province, particularly in the region of Guilin (Croom 1999). Entire families of tens of thousands of individuals ranging from the young through the elderly consume the aqueous extract in food daily. Croom (1999) noted that there is no evidence of any associated health issue with the consistent and daily use of Luo Han Guo over entire lifetimes.

While the fruit's traditional uses include various ethnomedical applications, as noted earlier, scientific evidence to support these putative benefits is lacking. It is clear that the primary use of the Luo Han fruit is based on its sweetness and consequent value as an ingredient in tea and other beverages.

4.7.1.2. Availability and Consumption of Luo Han Guo in the U.S.

The *Guangxi History of Chinese Medicine* (1963) includes a detailed record of harvested Luo Han fruits being prepared for export to Japan and the United States in 1885. There is evidence of importation into the United States by immigrants from Canton living in California and New York, many of whom were using it on a daily basis while laboring on the transcontinental railroad.

The earliest report of Luo Han Guo in a U.S. publication is the report by Groff and Ho (1938), which stated "... during a visit in 1917 to the United States Department of Agriculture, to botanist Dr. Frederick Coville's office, I (Groff) was shown a Luo Han fruit obtained from a local Chinese store in Washington, DC. It was purchased by Dr. Coville and Dr. Walter T. Swingle." Seeds from *Siraitia grosvenorii* fruits purchased in a San Francisco Chinese store were also included in Swingle's original botanical description of the species in 1941 (Swingle 1941). Similarly, Hussain et al. (1990) noted that the Luo Han samples studied had been purchased in Chicago's Chinatown.

Since 1980, Luo Han fruit and products containing the material, predominantly of Chinese and Japanese manufacture, have freely entered the European and American markets as foods. Although millions of Luo Han fruit are consumed worldwide each year, Luo Han fruits in Europe and the United States are mostly sold in Chinese grocery and herb stores.

4.7.2. Use of Products Derived from the Luo Han Fruit

Luo Han fruit is harvested from September through the end of November. In order to maintain a supply of fruit year-round and allow transport of the fruit to distant markets, it is common for the fruit to be dried, and this is how Luo Han fruit usually appears in Chinese groceries. The fruits are slowly dried in ovens; the drying process preserves the fruit and removes most of the objectionable flavor of the fresh fruit, which is associated with volatile components. Unfortunately, the drying also may cause the formation of bitter, astringent flavors due to production of melanoidins. These flavors limit the use of the dried fruits and dried fruit extracts to the preparation of dilute teas and soups and products to which sugar, honey, and the like are added.

In addition to the process used to produce PureLo®, several other methods have been developed to make a useful sweetener from the Luo Han fruit, and Luo Han extracts are currently used as ingredients in a great variety of food and dietary-supplement products in the U.S. and internationally. For example, one such process was patented in 1995 by the Procter and Gamble Company (Downton et al. 1995). As described in the patent application, in the P&G process, the fresh fruit is picked before ripening and allowed to complete its ripening during storage so that processing begins with the just-ripe fruit. The peel and seeds are then removed and the mashed fruit becomes the basis of a concentrated fruit juice or puree that can be used in food manufacturing. Further processing involves using methylene chloride, cation exchange resins, and pectinase to remove volatile and off-flavor components, and the addition of an acid to lower the pH.

Numerous sweeteners derived from Luo Han Guo by this and other similar processes that isolate the sweet compounds are now readily available for manufacturing and for kitchen use, and numerous products are already on the market in China and other countries, including the U.S. The PureLo® process is unique in using no solvents other than water.

4.7.2.1. Non-U.S. Luo Han Products

In China, Luo Han Guo was listed in the initial group of food derivatives that can be safely used as dietary supplements by the Administration of Chinese Medicine of the Ministry of Hygiene in 1987. In 1995, Luo Han mogroside was officially admitted as an approved food in China for use in beverages, foods, and confectionaries (*Food Usage Hygienic Standards*, GB 2760-1996 (1997 Amendment), p 94).

Luo Han fruit, "Rakanka" in Japanese, ranks third in popularity as a sweetener in Japan. "Rakanka" is an approved food in Japan found in beverages, foods, confectionaries, oral care products, and OTC products (*Health Industry Directory 2001-2002*, p 837; Category: Staples of raw materials approved as foods). According to confidential information provided by BioVittoria, exports of Luo Han fruit juice extracts and concentrates to Japan exceed 7 metric tons annually.

In Australia/New Zealand, Luo Han material is on the MedSafe list of ingredients approved for use in medicines and dietary supplements. (*Australian Department of Health and Aging, Therapeutic Goods Administration "Substances that may be used as "Listed" medicines in Australia.* September 4, 2004, page 44).

Many Luo Han products are currently sold in China, including teas, other beverages, grain products, flavors, and cough syrups and drinks. Some examples are:

- Zhizhonghe Luo Han Guo Beverage (Zhizhonghe Company Ltd,)
- Dayinxiang Luo Han Guo Tea (Shantou Great Impression Co., Ltd.)
- Luo Han Guo Food (Yongfu Technology Bureau)
- Luo Han Guo & Ginseng Grains (GB Luorensheng)
- Luo Han Guo Tea (Qingfutang Co.)
- Jin Shangzi Bai Cao (Luo Han Guo Tablet) (Top Fragrance Enterprise Ltd.)
- Luo Han Guo Tea (Guilin Songda Food Ltd)
- Luo Han Guo & Ginkgo Tea (Guilin Songda Food Ltd)
- Luo Han Guo Beverage (Guilin Songda Food Ltd)
- Tianduo Luo Han Guo Food (Tianduo Food Ltd.)
- Lim On Tong Pei Pa Koa (Luo Han Guo Flavor) (Kingto Lim On Tong)
- Luo Han Guo Paste (Huarentang Ltd.)
- Luo Han Guo Drink (Yipeitong Ltd.)
- Luo Han Guo Beverage (BioValley)
- Luo Han Guo Instant Beverage (Tea Plum GB)
- Luo Han Guo Tea (Guilin Shun Chang Food Ltd)
- Luo Han Guo & Ginseng Tea (Guilin Shun Chang Food Ltd)
- Luo Han Guo & Wolfberry Tea (Guilin Shun Chang Food Ltd)

- Luo Han Guo Beverage (Guilin Shun Chang Food Ltd)
- Luo Han Guo Cough Syrup (Yong Fu Pharmaceutical Factory)
- Luo Han Guo Cough Beverage (Yong Fu Pharmaceutical Factory)
- Premium Luo Han Guo & Glossy Ganoderma Tea (Guilin Guilong Food Factory)
- Premium Luo Han Guo & Ginseng (Guilin Guilong Food Factory)
- Premium Luo Han Guo & American Ginseng Tea (Guilin Guilong Food Factory)
- Luo Han Guo Sweet-Scented Osmanthus Tea (Guilin Grocery Food Ltd.)
- Specially-Made Luo Han Guo Tea (China Guangxi Luo Han Guo)
- Multi-Ingredient Luo Han Guo Cough Beverage (Guangxi Jin Hai Tang
- Pharmaceutical Ltd.)
- Multi-Ingredient Luo Han Guo Cough Beverage (NanNing WeiWei Pharmaceutical Ltd.)
- Multi-Ingredient Luo Han Guo Cough Beverage (Guangxi TianTian Le Pharmaceutical Ltd.)

Luo Han products are available in other countries also, such as the following:

- Thailand: Instant Luo Han Kuo (Khao-La-Or Laboratories Ltd.)
- Japan: LaKanTo Cooking Sugar (Luo Han Guo Sugar) (Saraya Co., Ltd.)
- Germany: Luo Han Guo Tabletten (Energia Vital)
- Germany: Kwei Feng Kräuter Tee (Chinesische Lebensmittel)
- Norway: Luo Han Guo Drink (OsloFoodie)

4.7.2.2. Luo Han Products Sold in the U.S.

As noted earlier, Luo Han fruit has been imported and sold in the U.S. since the late 1800's with the migration of Asian workers who were building the early railroads. More recently ingredients and finished products have been introduced and sold in all market sectors including sales over the Home Shopping Network, through catalogs, and at retail. Products currently using Luo Han include beverages, foods, table top sweeteners, and oral care products. These products have been imported under the following Tariff numbers:

- Import Tariff No. US HTS # 1302 19 90 40
- FDA Product No. 24JVH20 (cucumber juice in vacuum package)

Several companies offer Luo Han Guo extracts as dietary supplements, claiming a variety of benefits similar to those attributed to Luo Han in Chinese folk medicine. However, in addition to the absence of data to support these claims, the companies selling the extracts as dietary supplements recommend doses of 3.5 g or higher—a level nearly 10 times higher than that estimated to occur at the 90th percentile from the intended uses of PureLo®. Most of the uses of existing Luo Han extracts in the U.S., however, are as a flavor modifier and sweetener rather than as a dietary supplement ingredient.

In their survey of sweeteners derived from plants, Kinghorn and Soijarto (2002) noted that, "Soft drinks incorporating extracts of *Siraitia grosvenorii* (Swingle) Lu & Zhang (Cucurbitaceae) fruits (also known as "Luo Han Kuo"), containing sweet

cucurbitane-type triterpene glycosides such as mogroside V, are now on the market" in the U.S. In addition, numerous other beverages as well as sweeteners intended for addition to foods are available in the U.S., including the following, intended as a representative rather than exhaustive listing:

- SlimSweet (Luo Han fruit concentrate) (TriMedica International, Inc.)
- Luo Han Sweet (Jarrow Formulas)
- Sweet Sensation Luo Han Guo Powder (VitaSprings)
- Luo Han Kuo Health Drinks (Longjiang River Health Products LLC)
- English Toffee Dessert Tea (Hain Celestial Group)
- Tonic Alchemy (pH Advice)
- Neway All Purpose Sweet Sensation Natural Luo Han Guo (Newayceutical)
- KAL Pure Stevia Plus Luo Han (National Supplement Center)
- Sweet & Slender (Seedman)
- Luo Han Liquid Extrack by Kal (4 All Vitamins LLC)
- Luo Han Guo Fruit Syrup Sugar Alternative (Organic Direct)
- Lo Han Kuo Beverage (KT Botanicals)
- HerbaSweet (HerbaSway Laboratories LLC)
- SweetLIFE (Healthy Highway)
- Forte Juice w/ Acai, Pomegranate, Blueberry, & Luo Han Guo (911 Health Shop)
- Natural Luo Han Guo Fruit Syrup (VitaSprings)
- Magic Fruit (Natural Ways)
- Figure 8 Shakes (Figure 8)
- Sugar Not (Dixie USA Inc.)

Over the past decade, one manufacturer alone has exported 21.6 metric tons of a Luo Han fruit concentrate similar to PureLo® to the United States and another 2.5 metric tons to Japan and other countries (confidential information provided by BioVittorio). The export records provided are summarized in Table 10.

Table 10. Exports of Luo Han Fruit Juice Concentrate by One Manufacturer.

Year	Total Exports	Exports To U.S.
	Metric Tons	
1996	2.0	1.8
1997	2.2	1.9
1998	2.4	2.2
1999	2.6	2.3
2000	2.0	2.0
2001	2.2	2.2
2002	2.5	2.2
2003	3.0	2.6
2004	5.2	4.4
Source: BioVittoria		

As can be seen in Table 10, annual exports of Luo Han fruit juice concentrate to the U.S. have been increasing, from 1.8 metric tons in 1996 to 4.4 metric tons in 2004.

4.7.2.3. FDA Responses to Lo Han Products Intended for Sale in the U.S.

Two New Dietary Ingredient notices have been filed with FDA under the provisions of the Dietary Supplement Health and Education Act (DSHEA) regarding the use of Luo Han products as dietary supplement ingredients. In the first of these, dated March 4, 1996, HerbaSwy Laboratories, Inc., notified FDA of their intention to use "Lo Han Kuo Extract," identified only as a "fruit extract of *Siraitia grosvenorii* S," as an ingredient in dietary supplements with an addition level of 60-300 mg in a fluid supplement (FDA 1996). FDA filed this notice without comment, implicitly accepting that the intended use (with an estimated daily intake similar to the potential intake of Luo Han fruit concentrate) is safe over an extended period of consumption.

The second notice was filed by Nature's Marvel International on October 6, 1999 (FDA 1999). "Lo Han Kuo Fruit Extract," identified as a "fruit extract of *Siraitia grosvenorii* (swingle) C. Jeffrey," was intended for use as a sweetener. The FDA review concluded that this use is not congruent with the definition of a dietary ingredient provided by DSHEA, and thus rejected the notice. FDA did not address the safety of the proposed use.

4.8. Equivalence of PureLo® and Traditionally Prepared Luo Han Fruit

PureLo® Luo Han fruit concentrate contains only components found in the fresh fruit. The primary traditional use of the fruit involves boiling dried material in a filtered water solution to release the desired components. The PureLo® process differs in that it begins with fresh-picked fruit rather than traditional smoke-dried preserved fruits to avoid off-flavors that may be produced by this treatment. However, PureLo® Luo Han

fruit concentrate is also based on boiling the fruit and then filtering the resulting decoction.

In addition, since drying coagulates and denatures a significant amount of the pectin and trace protein in the fruit, creating off-tastes, the PureLo® process includes a resin clarification step to remove the pectin, protein, and sugars.

It was noted above that, in addition to its ethno-medical uses as a cooling beverage, Luo Han fruit is simmered into a thick syrup and saved for use as a sugar substitute in cooking. The processing of Luo Han fruit to produce PureLo® Luo Han fruit concentrate (shown earlier in Figure 4) parallels this traditional preparation (Figure 6).

Figure 6. Production Steps for PureLo® and Traditional Luo Han.

PureLo	Traditional Luo Han
[no equivalent]	Dry fruits
Crush/shred fruits	Cut up fruits
Decoct in boiling water	Decoct in boiling water
Ultrafilter	Decant decoction
Cool	Cool
Filter with macroporous resins	Strain with sieve or cloth
Condense by heating under vacuum	Condense by simmering
Spray dry	[no equivalent]

As is evident, the process by which PureLo® Luo Han fruit concentrate is produced closely parallels the traditional method, differing primarily in being a large-scale commercial process rather than a home preparation method. Further, the PureLo® process does not lead to chemical alteration of the material, and thus the PureLo® dried Luo Han fruit concentrate is equivalent to the traditional dried fruits and syrups made from the fruit and used for sweetening.

PureLo® Luo Han fruit concentrate is compositionally similar in its mogroside V content to the traditional preparations of Luo Han Guo that have been used in a wide variety of beverages, confections, and meat dishes throughout Asia.. As discussed above, these traditional preparations are commonly consumed by all sectors of the population in China, Japan, Malaysia, Singapore, and in the Asian populations within the U.S., Canada, and Australasia.

The compositional equivalence of PureLo® Luo Han fruit concentrate to traditionally prepared Luo Han fruit decoction is demonstrated in the HPLC traces shown in Figure 7. Prior to the analysis, the PureLo® sample was diluted to lower the height of the elution peaks in order to provide visual separation when the traces were overlaid. During approximately the first 10 minutes, the trace of traditional preparation shows a number of phenolic peaks. Since most of the phenolic compounds are removed from PureLo® when the product is filtered through macroporous resin, these peaks are absent from the PureLo® trace. After this point, the traces are essentially identical through the full 50 minutes during which elution occurs. The large peak, at a retention time of 33.3 minutes, is mogroside V.

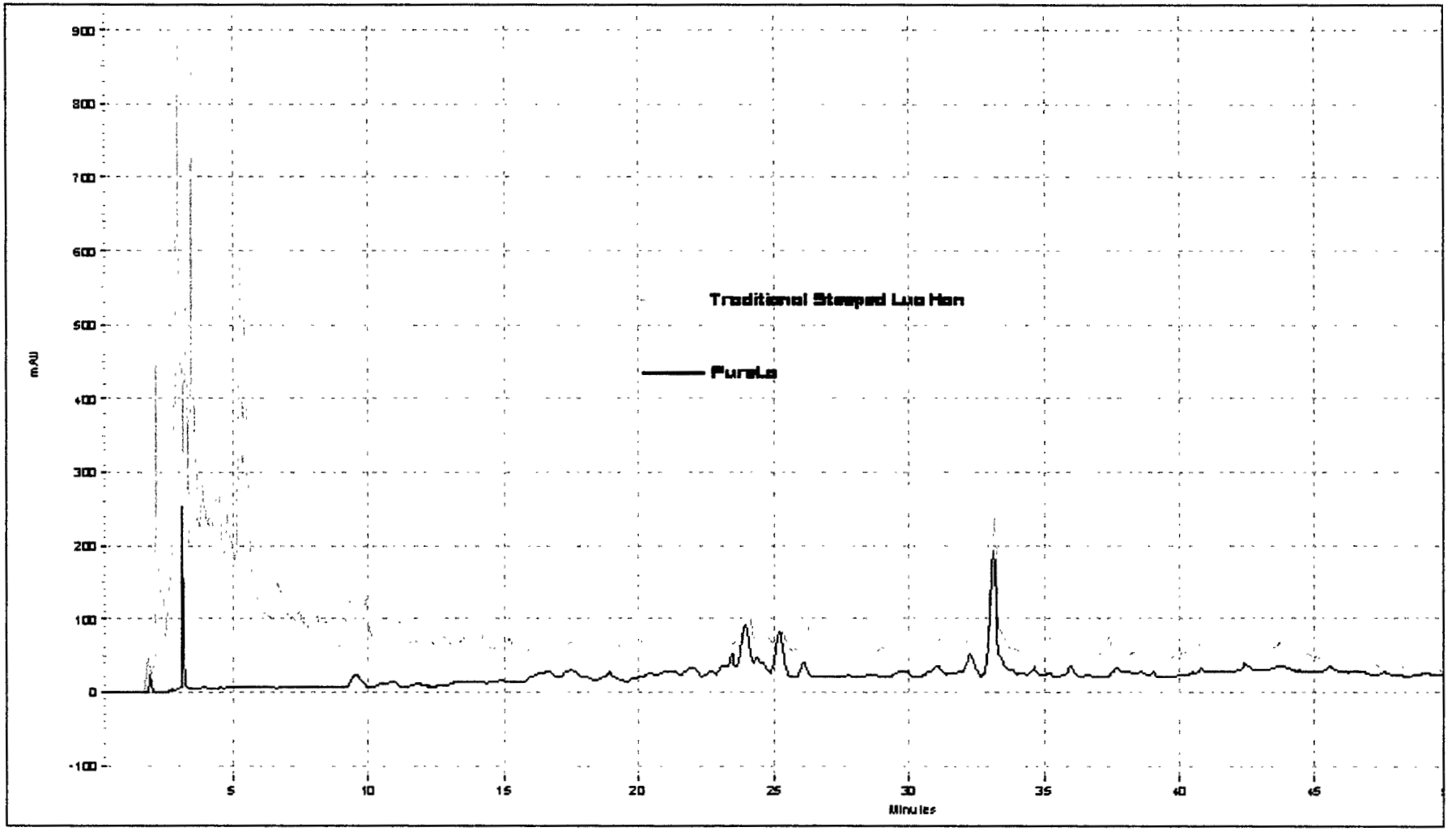

Figure 7. HPLC Traces of PureLo® and Traditional Luo Han Guo

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5. SAFETY ASSESSMENT AND GRAS DETERMINATION

5.1. Introduction

This chapter presents an assessment that demonstrates that BioVittoria's PureLo® brand Luo Han fruit concentrate is a food that is safe and GRAS for consumption as a stand-alone sweetener, as a component of a sweetener blend, or as an ingredient to be added to other foods as a taste modifier or to provide sweetness. This safety assessment and GRAS determination entail two steps. In step one, the safety of Luo Han fruit concentrate under its intended conditions of use is demonstrated. Safety is established by showing that PureLo® brand Luo Han fruit concentrate is a food product that is substantially similar in composition to preparations of the fruit that have been consumed both in China and in the U.S. for decades and that the Luo Han fruit is processed to produce PureLo® Luo Han fruit concentrate using methods that parallel those traditionally used to produce Luo Han concentrates. It is further shown that the intake of mogrosides and other Luo Han constituents from the intended use of Luo Han fruit concentrate does not exaggerate the level of intake that results from traditional consumption of the raw or dried fruit or preparations made from this fruit. Further evidence of safety is provided by the widespread use in the U.S. and internationally of extracts of the Luo Han fruit juice as sweeteners and as dietary supplement ingredients, the latter at far higher levels of intake than are expected to result from the intended uses of Luo Han fruit concentrate. Finally, published subacute and subchronic oral toxicity studies show no adverse effects at exposures to Luo Han fruit concentrate of more than 1000 times the intake expected to result from Luo Han fruit concentrate's intended uses, thus providing strong experimental evidence demonstrating the safety of Luo Han fruit concentrate for its intended uses.

In the second step, Luo Han fruit concentrate is determined to be GRAS by demonstrating that the safety of this substance under its intended conditions of use is based on generally available information and is generally recognized among qualified scientific experts.

The regulatory framework for establishing whether a substance is GRAS in accordance with Section 201(s) of the FDCA is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under 21 CFR §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under 21 CFR §170.30(c). This GRAS determination is based on scientific procedures; while experience derived from common use prior to 1958 and from more recent uses of Luo Han juice extracts provides considerable assurance of safety, results of *in vitro* testing of cytotoxicity, genetic toxicity assays, and repeated-dose animal toxicity studies provide the primary basis for both safety and GRAS.

A GRAS determination requires that the evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components: 1) the data and information relied upon to establish the scientific element of safety must be generally available; and 2) there must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

5.1.1. EDI of Luo Han Fruit Concentrate

As was discussed in Section 2, the primary use of Luo Han fruit concentrate is as a sweetener, most commonly as one component of a sweetener blend. Luo Han fruit concentrate alone or blends containing Luo Han fruit concentrate are thus alternatives to sucrose and other sugars or to other intense sweeteners and may be used in formulated foods to replace such sweeteners or as tabletop sweeteners to be added to foods by the consumer as alternatives to table sugar. In Section 3, it was estimated that complete replacement of all intense sweeteners by Luo Han fruit concentrate would potentially result in a 90th percentile daily exposure as high as 10 mg/kg bw/day for some population segments, with a 90th percentile daily intake for the general population (the EDI) of 6.8 mg/kg bw/day (see Table 7).

There are no other significant sources of mogroside V or the other mogrosides found in Luo Han fruit concentrate in the U.S. diet, except for the few individuals consuming traditional decoctions of dried Luo Han fruit or one of the existing Luo Han products that compete with PureLo® brand Luo Han fruit concentrate for the same sweetening uses. Thus this EDI represents the total intake of Luo Han fruit concentrate and its primary constituents.

5.1.2. Safe Levels of Intake of Luo Han Fruit Concentrate

The FDA has not previously raised any safety concerns regarding consumption of Luo Han fruit or extracts from Luo Han fruit such as those based on the process patented by Procter & Gamble. There are a number of such products on the market in the U.S. and elsewhere with no reports of adverse effects. HerbaSwy Laboratories notified FDA of the intended use of "Lo Han Kuo Extract" as a New Dietary Ingredient with a planned use level of up to 300 mg in a fluid dietary supplement (FDA 1996). FDA filed this notice without comment, accepting that this level of intake has been shown to be safe.

The production of PureLo® Luo Han fruit concentrate does not differ remarkably from methods used to produce other fruit-derived beverages. It was further shown that PureLo® Luo Han fruit concentrate is compositionally similar to traditionally prepared dried Luo Han decoctions, and thus does not pose safety concerns. The concentration that occurs in Luo Han fruit concentrate is not extreme: it contains about 30-35% mogroside V, virtually identical to the concentration found in the traditional decoction. Dried fruit has been found to contain on average about 0.5% mogroside V by weight, thus, the estimated daily intake of mogroside V from the intended use of Luo Han fruit concentrate as a flavor modifier and sweetener is approximately equal to the amount that would be obtained from about 24 g of the dried fruit, well within a reasonable serving size.

Three repeated-dose oral toxicity studies of PureLo® Luo Han fruit concentrate or a closely matched competitive product have been completed and published. In all studies, the NOAEL was the highest dose tested. In a subacute (28-day) feeding study of PureLo® in SD rats, the NOAEL was 7071 mg/kg bw/day; in a subchronic (90-day) study of PureLo® in dogs, the NOAEL was 3000 mg/kg bw/day; and in a subchronic study of a similar Luo Han fruit concentrate in Wistar Hannover rats, the NOAEL was 2520 mg/kg bw/day.

5.2. General Recognition of the Safety of Luo Han Fruit Concentrate

Because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of PureLo® Luo Han fruit concentrate for use as a table-top sweetener and for direct addition to foods has been made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and John A. Thomas, Ph.D. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have critically reviewed and evaluated the publicly available information summarized in this document, including the potential human exposure resulting from the intended use of Luo Han fruit concentrate, as well as other information deemed pertinent, and have individually and collectively concluded:

> *PureLo® Luo Han fruit concentrate has been sufficiently characterized to ensure a wholesome and food-grade product. Ingestion of Luo Han fruit concentrate from the intended uses results in intakes of Luo Han fruit concentrate that remain within safe limits established by both the history of use of Luo Han fruit and published safety studies. Therefore, the intended use of Luo Han fruit concentrate meeting the specifications described in this GRAS monograph is safe.*

It is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion. Therefore, PureLo® Luo Han fruit concentrate is safe and GRAS by scientific procedures for use as a flavor modifier and sweetener as described herein.

The signed Conclusion of the Expert Panel is attached as Appendix III.

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APPENDIX I
Analytic Methods

Analytical Procedure for Measuring Mogroside Content in Luo Han Fruit Concentrate

The sweet characteristic of Luo Han is due to the presence of mogrosides, which are triterpene glucosides. The aglycone of these triterpenes is a terpene alcohol which produces a color reaction with a vanillin- sulfuric acid solution. Because the color reaction is not affected by the glucoside content, this reaction can be used to measure the mogroside content of PureLo® Luo Han fruit concentrate.

Vanillin- Sulfuric Acid Solutions.

To make a 10% vanillin-sulfuric acid solution, 2.5g vanillin is added to concentrated sulfuric acid and made up 25ml volume.

To make a 50% (v/v) sulfuric acid solution, take 50ml concentrated sulfuric acid and slowly add it to 50ml distilled water, stirred thoroughly. Cool to room temperature and put it into a 100ml flask.

Sample Analysis for Total Mogroside

40 mg of sample is placed in a 25 ml flask to which 15 ml methanol is added, heat to 70°C in a water bath until the sample is completely dissolved. The flask is removed from the water bath cooled and methanol is added to make to 25 ml volume. Take 0.50 ml of this sample solution and place in a test tube, boil and evaporate till dry; add 0.50 ml 10% vanillin - sulfuric acid solution, shake, cool on ice and then add 50% sulfuric acid solution, shake until the sample is completely dissolved; heat in 60°C water bath for 20 minutes then immediately cool on ice for 10 minutes; determine the color of the solution by measuring the absorbance at 600 nm wavelength, and then calculate the total mogroside content.

Calculation for Total Mogroside Content

$$\text{Total mogroside \%} = \frac{A \times V}{K \times G}$$

Notes:
A: Sample absorbance
V: Sample solution volume, which is 25 ml
K: Constant, 8.15
G: Sample weight (g)

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Mogroside V Analysis
Mogroside V is analyzed using liquid chromatography as follows:

Chromatographic column: Shodex Asahipak NH_2 P 5μ (4.6 mm diameter X 250 mm)
Wavelength: 203nm Flow speed: 1.0 ml/min
Temperature: 40°C Stop time: 40 min

Acetonitrile (or ethanenitrile, CH_3CN) (Chromatography): water (twice diluted water) = 74:26

Dissolve 40 mg of sample in methanol solution (7 methanol:3 water), to a volume of 25 ml. Make just before use.

Sample input amount: 20 μl

Setting of the chromatography: Paper feeding speed: 96 cm/h; deduction: 500 mv

Sample preparation: heat sample to 100°C for 2 hours, place in the desiccator and weight 40 mg. Add in 7.5 ml distilled water and 8 ml methanol solution, shake until completely dissolve, fill in methanol until reach 25 ml.

Instrument operation: vacuum filtrate water and acetonitrile separately, start the instrument and deoxidate them separately. Put them into the chromatographic column; wait until the baseline is stable. Switch on the chromatography, start putting in samples after the baseline has kept 30 mm stable. Filtrate the sample with Jane's filter absorb 40 μl sample, with strictly no bubble in it, and then put it into the instrument, press the start button at the same time. Observe the next 40 mm. If the sample peak is symmetry and sharp, and if after all peaks the baseline can return to the start point, then the test is successful.

Result calculation: draw a straight line to connect the start point and the end point of the graph, measure the peak high of Mogrosides and the width of its half peak using vernier caliper, multiply these two measurements and compare with the contrast and calculate the mogrosides V content.

Product Appearance

Color, shape and residues
Place the sample on a clean, white paper, inspect its color without direct sun shine, shape and dross (refer to GB/T 5492-2008)

Product Taste
Solute product and make 0.1% solution, and taste (refer to GB/T 5492-2008)

Product Fineness
Put products of certain weight through bolter, the remnant amount should be less than 5% (weight ratio) (refer to GB/T 5507)

Product Solubility
Weigh 1.0 g of product, place it in a clean and dry breaker, solute with 100 ml water, (could be heat within water), dry the outside of the breaker, relocate it to special work table and observed the solubility with sufficient light.

pH Test
Make 0.1% solution and test it with pH meter.

Heavy Metal Test
Arsenic: refer to GB/T 5009.11-2003
Lead: refer to GB/T 5009.12-2003
Copper: refer to GB/T 5009.13-2003

Microbiology
Aerobic Plate Count: refer to GB/T 4789.2-2008
E. Coli: refer to GB/T 4789.3-2008
Total Yeast and Mold: refer to GB/T 4789.15-2003
Pathogenic microorganisms: refer to GB/T 4789.4,5,10-2008

APPENDIX II

CHROMADEX REPORTS

ChromaDex™

10005 Muirlands Blvd., Suite G | Irvine, CA 92618
Phone: (949) 419-0288 | Fax: (949) 419-0294
www.chromadex.com

Analytical Results Sheet

Customer:	BioVittoria Limited	Report Number:	CDXA-ARS-4464-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD28606
Sample Name:	PureLo		
Sample Lot:	G-2009000	Date Received:	02-June-09
CDXA Number:	CDXA-09-1623	Purchase Order:	N/A
Assay:	Sugars Analysis by GC	Date of Report:	16-Jun-09
Part Number:	CDA-00100274-ARS	Page:	2 of 2
Method:	AOAC 977.20	Test Location:	Sub20

Analyte	Units	Spec.	Result	Reporting Limit
Fructose	%	N/A	ND	0.1
Glucose	%	N/A	ND	0.1
Sucrose	%	N/A	2.78	N/A
Maltose	%	N/A	ND	0.1
Lactose	%	N/A	ND	0.1
Total Sugars	%	N/A	2.78	N/A

*N/A = Not Applicable

Approved: Kimberly Eastman

Digitally signed by Kimberly Eastman
DN: cn=Kimberly Eastman, o=ChromaDex, ou=CDXA, email=KimE@chromadex.com, c=US
Reason I am approving this document.
Date: 2009.06 16 16.46'47 -06'00'

Signed original on file at CDXA

ND – Not Detected
BRL – Below reporting limit (compound detected below RL)

ChromaDex™

10005 Muirlands Blvd., Suite G | Irvine, CA 92618
Phone: (949) 419-0288 | Fax: (949) 419-0294
www.chromadex.com

Analytical Results Sheet

Customer:	BioVittoria Limited	Report Number:	CDXA-ARS-4464-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD28606
Sample Name:	PureLo		
Sample Lot:	G-2009000	Date Received:	02-June-09
CDXA Number:	CDXA-09-1623	Purchase Order:	N/A
Assay:	Total Dietary Fiber	Date of Report:	16-Jun-09
Part Number:	CDA-00100306-ARS	Page:	1 of 2
Method:	AOAC 991.43	Test Location:	Sub20

Analyte	Units	Spec.	Result	Reporting Limit
Total Dietary Fiber	%	N/A	ND	0.1

*N/A = Not Applicable

Signed original on file at CDXA

ND – Not Detected
BRL – Below reporting limit (compound detected below RL)

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Analytical Test Report

Customer:	BioVittoria Limited	Report Number:	CDXA-ATR-1442-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD27787
Purchase Order:	-	Date Received:	16-Apr-09
Date of Report:	21-May-09	Test Location:	Boulder, CO

Assay: LC/MS Fingerprint on PureLo

Part Number: CDA-00100104-ATR

Prepared By: **James Traub** _____ _____
Analytical Chemist Date

Reviewed By: **Seth Nosel** _____ _____
Quality Assurance Date

Approved By: Steve Baugh Digitally signed by Steve Baugh DN: cn=Steve Baugh, email=steveb@chromadex.com, o=ChromaDex Analytics, ou=CDXA, c=US Date: 2009.05.21 17:15:19 -06'00'
Steve Baugh _____
Manager, Analytical Services Date

Signed original on file at CDXA

SUMMARY

- ***SAMPLE(S)***

	Lot #	CDXA #
PureLo	G-2009000	CDXA-09-1623

- ***RESULTS***

Table 1 - CDXA-09-1623

Analyte	Units	Spec.	Result	Reporting Limit
11-Oxo-Mogroside V	%	-	8.01	-
Mogroside V	%	-	34.9	-
Siamenoside I	%	-	1.95	-
Grosmomoside I	%	-	2.67	-
Total	**%**	-	**47.5**	-

Sample CDXA-09-1623 was analyzed by HPLC/MS and four Mogroside-related compounds were detected and quantified. Peaks in the sample were identified and assigned by HPLC retention time and UV spectra, and identity was then confirmed by mass spec against qualitative standards.

A calibration was performed for Mogroside V (the only available Primary grade standard at the time of testing) and all compounds were quantified using this curve.

No presence of other, unidentified Mogroside compounds was detected.

ANALYTICAL METHOD

- **STANDARD(S)** *All standards supplied by ChromaDex, unless otherwise specified.*

	Part #
Mogroside V	ASB-00013881

- **LABORATORY SUPPLIES**

Analytical Balance
Ultrasonication Bath
Assorted and Volumetric glassware
Syringes and Syringe Filters
HPLC/GC glass vials and caps

- **SOLVENTS AND REAGENTS**

Acetonitrile (ACN)
Methanol (MeOH)
Milli-Q Water

- **SOLUTION PREPARATION**

N/A

- **STANDARD PREPARATION**

Stock Standard Solution

Solution was prepared by weighing approximately 10 mg of standard into a 10 mL volumetric flask, bringing the flask to mark with methanol, and sonicating the solution for 10 minutes. Calibration standards were then prepared.

- **SAMPLE PREPARATION**

Customer Sample(s)

Sample was prepared by weighing approximately 100 mg of sample into a 100 mL volumetric flask, bringing the flask to mark with methanol, and sonicating the solution for 15 minutes. An aliquot was then filtered (0.45 µm PTFE) into an HPLC vial for analysis.

- ***INSTRUMENT PARAMETERS***

Instrument	Agilent 1100 Series HPLC System
Detection	UV-Vis
Mobile Phase A	Water
Mobile Phase B	Acetonitrile

Gradient Program

Time (min)	%A	%B
0.0	95	5
5.0	90	10
20.0	50	50
24.0	10	90
28.0	10	90
31.0	95	5
36.0	95	5

Column	YMC J'Sphere ODS-H80 250 x 4.6mm, 4µ
Flow Rate	1.750 mL/min
UV Detection	205 nm
Injection Volume	10 µL
Temperature	60 °C
Mass Spectrometer	Agilent 1100 Series Ion Trap
Ion Source	APCI
Ion Polarity	Positive
Target Ions	See Below

Compound	Agylcone Core
11-Oxo-mogroside V	439.2 *m/z*
Mogroside V	423.2 *m/z*
Siamenoside I	423.2 *m/z*
Grosmomoside I	423.2 *m/z*

DATA

- **FIGURES**

Figure 1: Mogrosides Mixed Standard (UV Chromatogram)

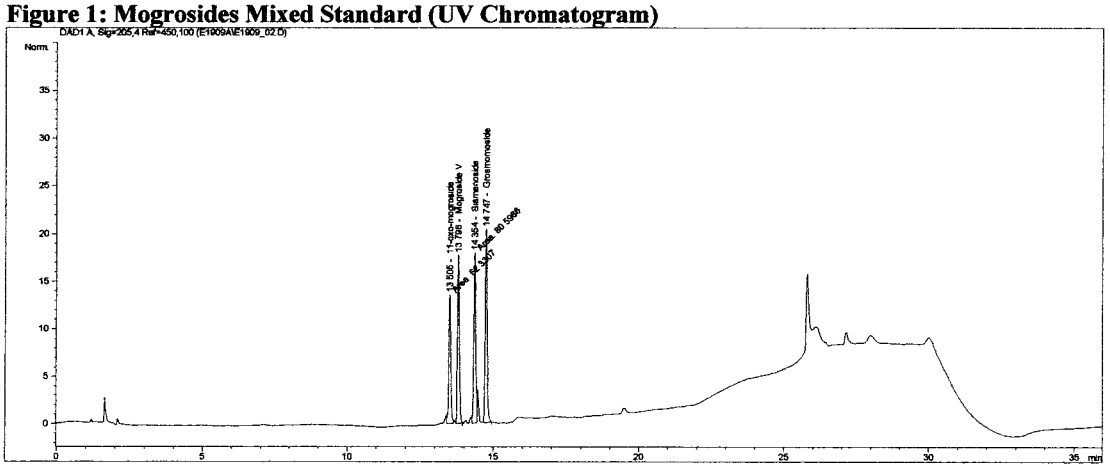


Figure 2: Mogrosides Mixed Standard – Enlarged (UV Chromatogram)

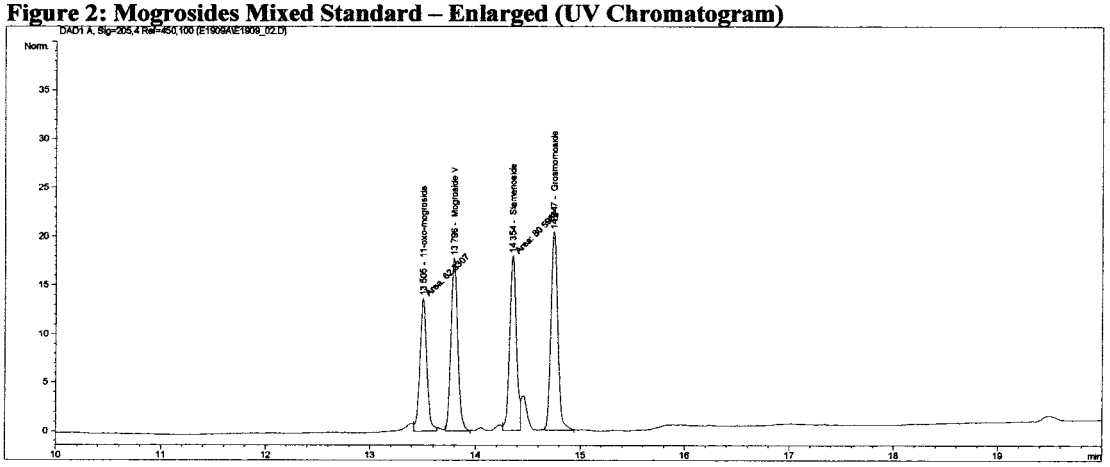


Figure 3: 11-Oxo-mogroside V Standard (UV Chromatogram)

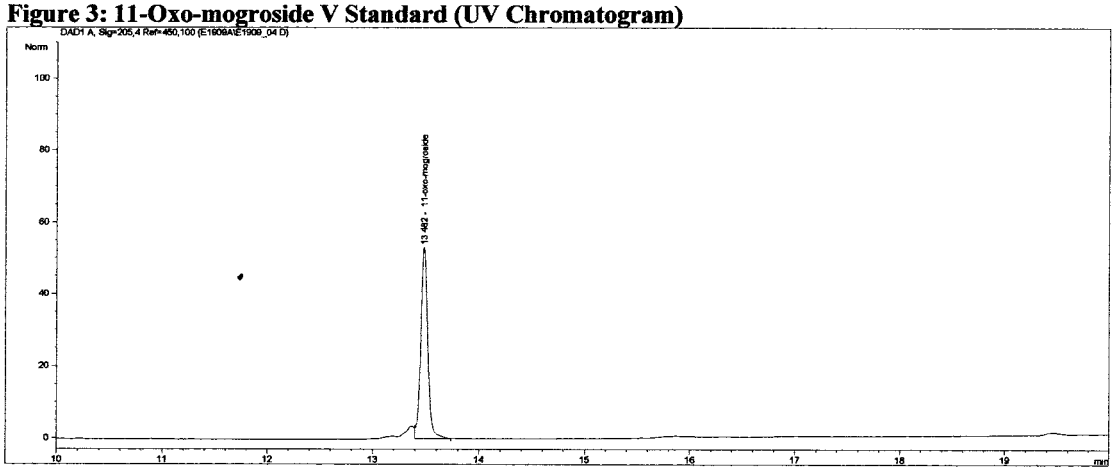


Figure 4: Mogroside V Standard (UV Chromatogram)

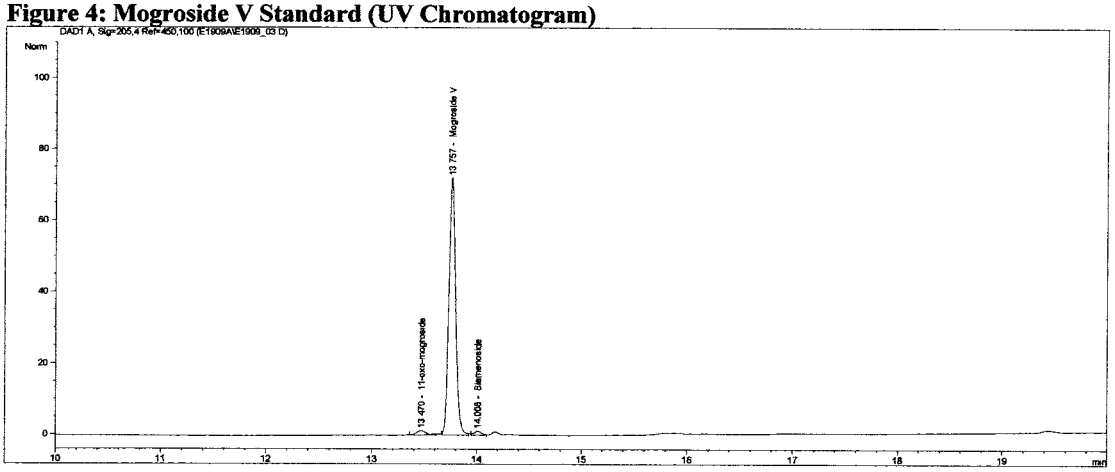


Figure 5: Siamenoside I Standard (UV Chromatogram)

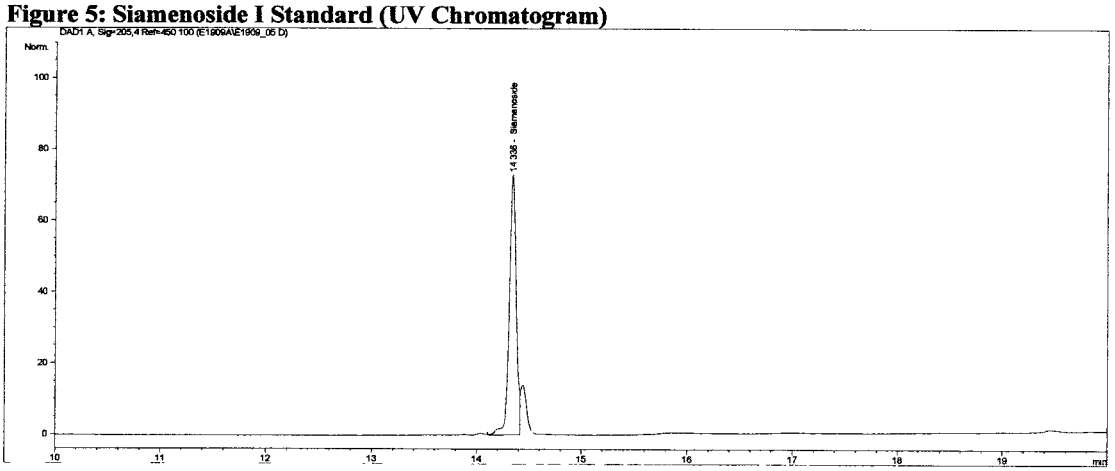


Figure 6: Grosmomoside I Mixed Standard (UV Chromatogram)

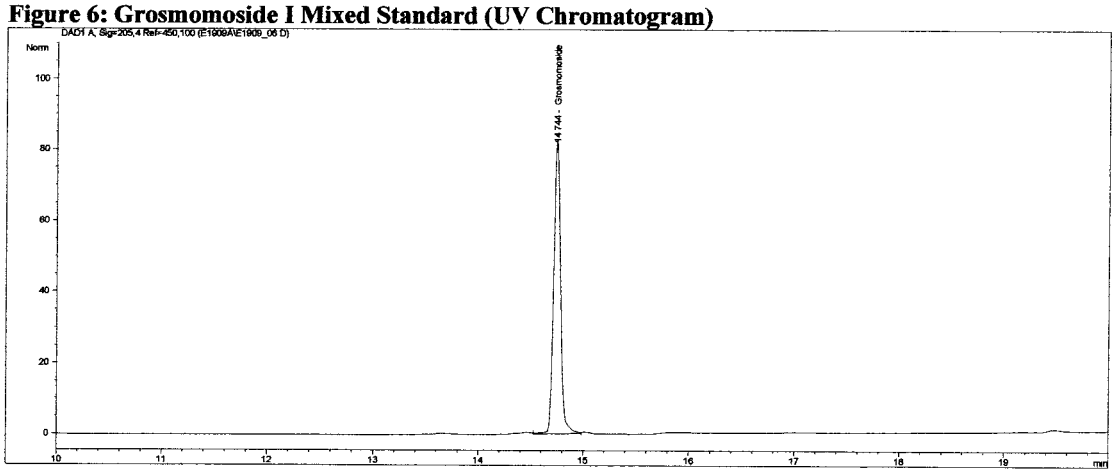


Figure 7: UV Spectra of Mogroside Standards

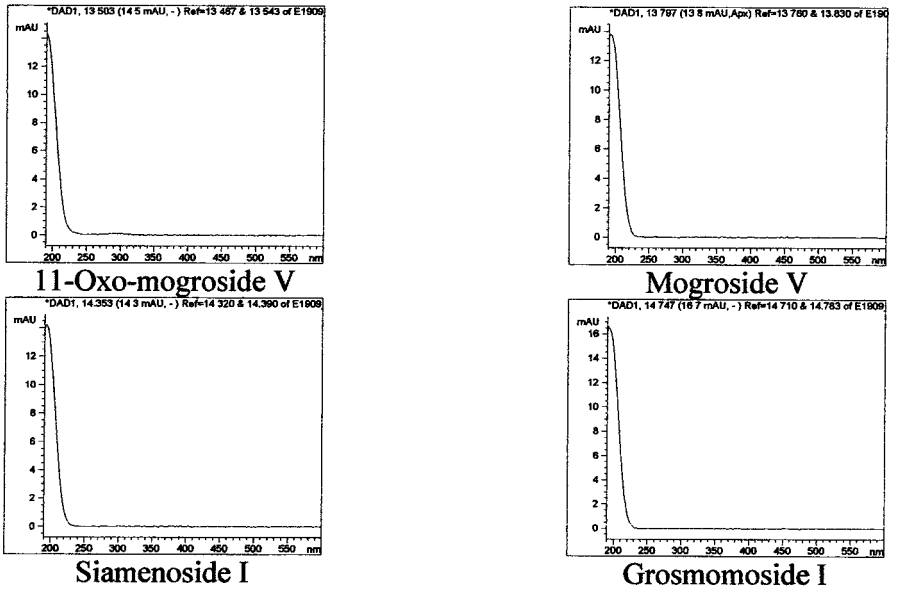


Figure 8: Mogrosides Mixed Standard (UV Chromatogram, Extracted Ion @ 423.2 *m/z*, Extracted Ion @ 439.2 *m/z*, Mass spectra of EIC peaks 1-4)

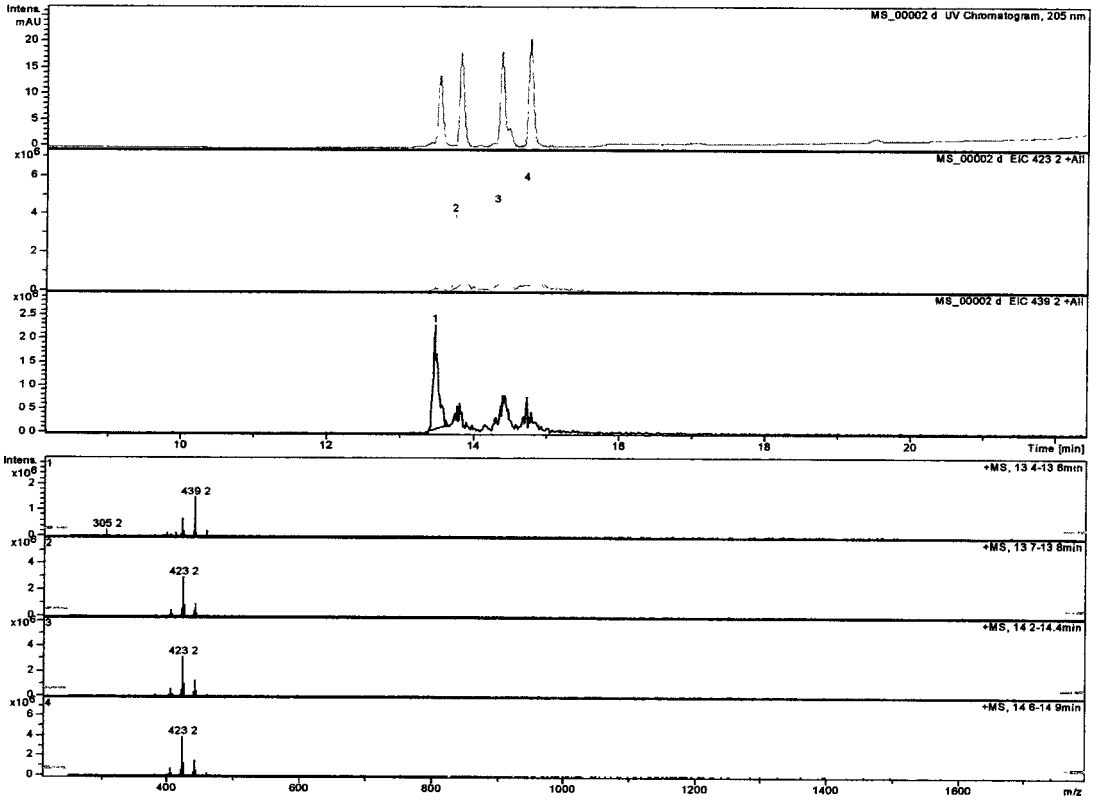


Figure 9: Sample CDXA-09-1623 (UV Chromatogram)

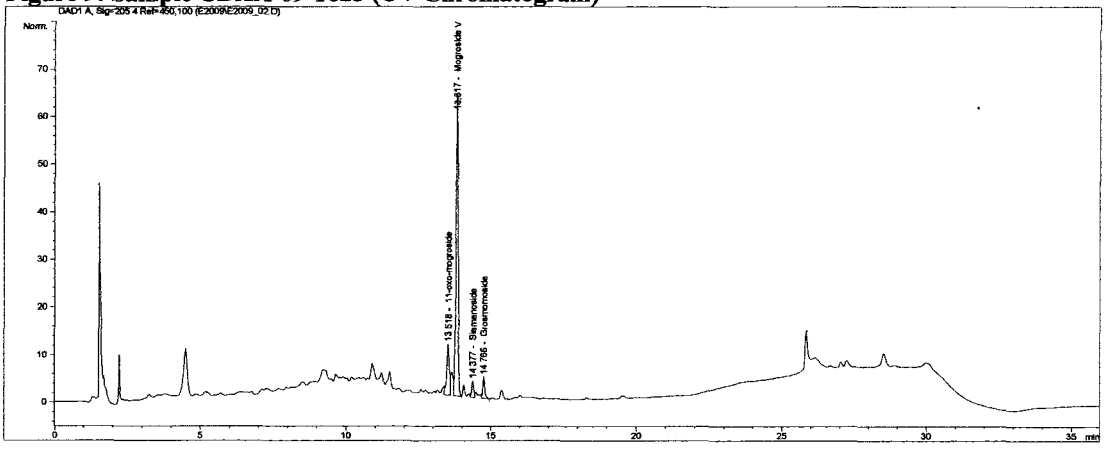


Figure 10: Sample CDXA-09-1623 – Enlarged (UV Chromatogram)

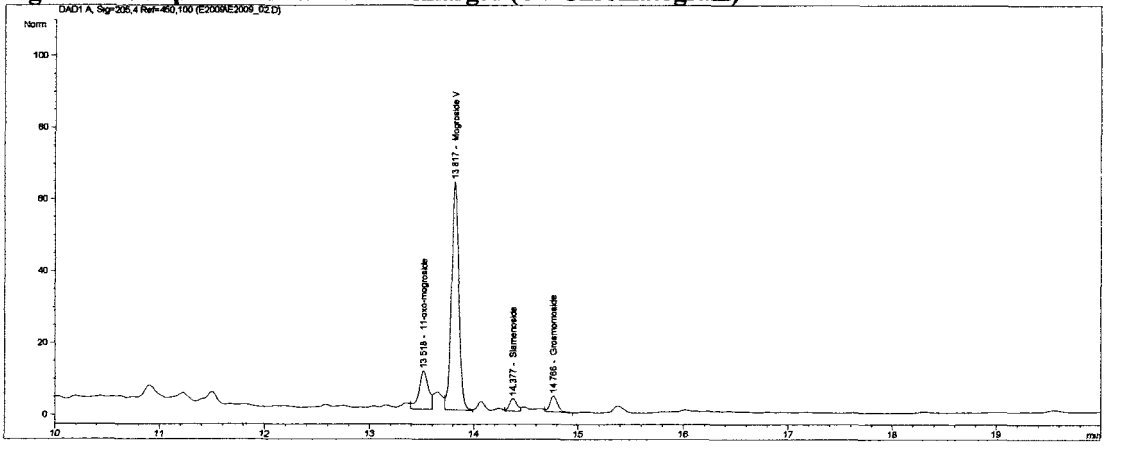


Figure 11: UV Chromatogram Overlay (Sample CDXA-09-1623—Dashed, Mogrosides Mixed Standard—Solid)

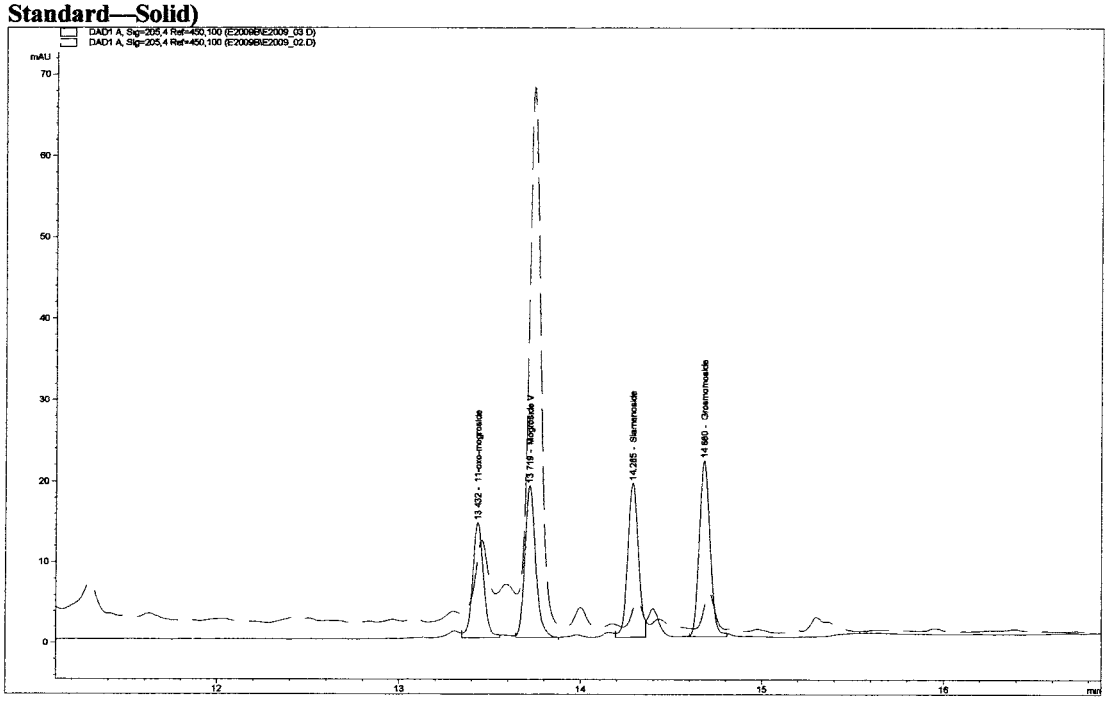
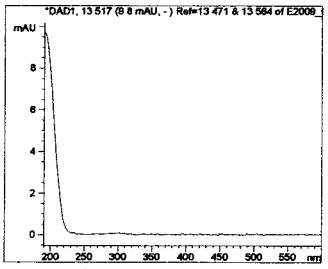
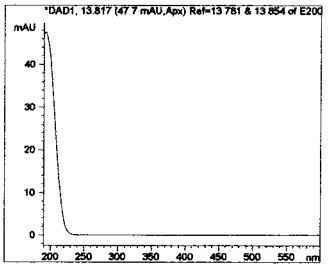


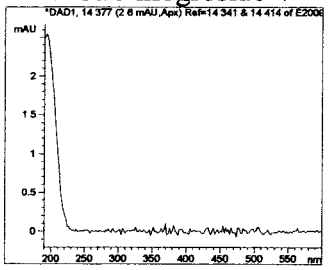
Figure 12: UV Spectra of Identified Mogroside Peaks in Sample CDXA-09-1623



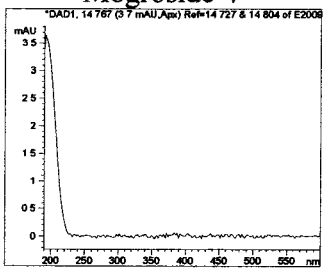
11-Oxo-mogroside V



Mogroside V

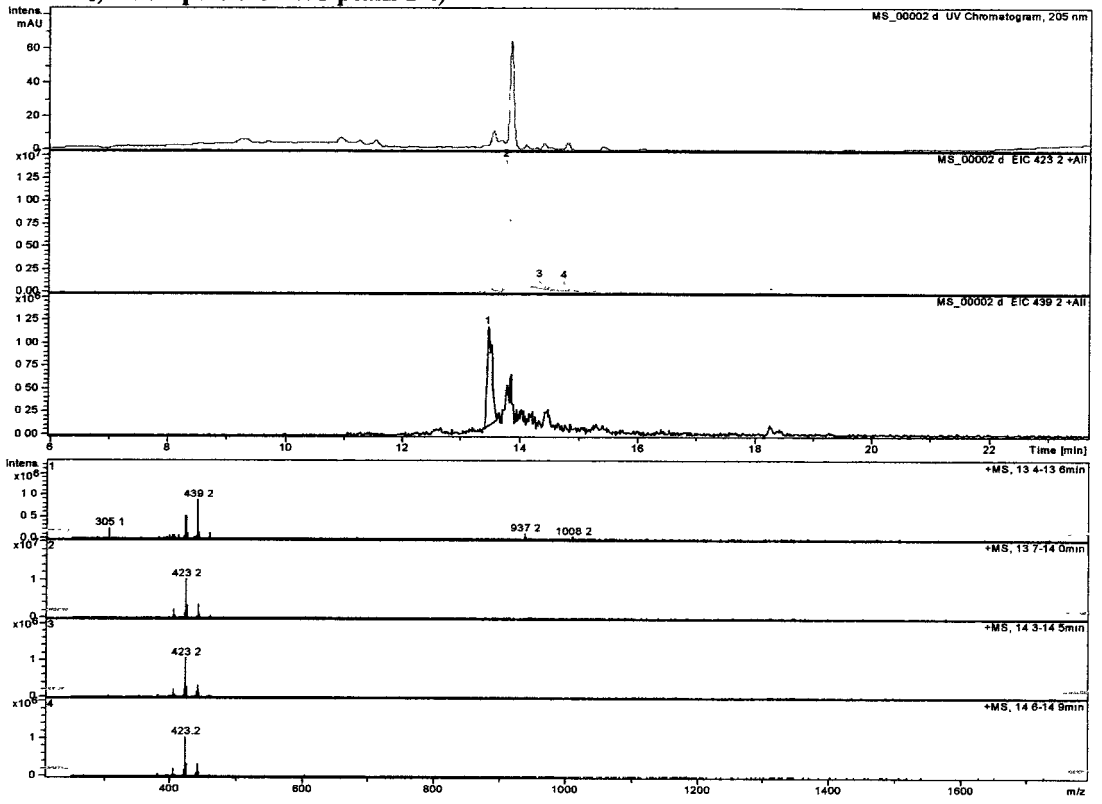


Siamenoside I

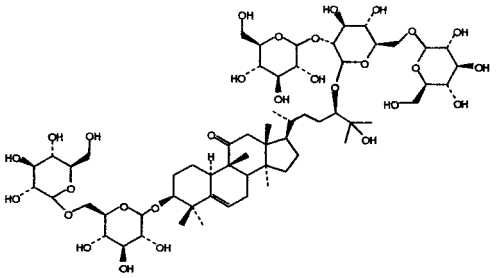


Grosmomoside I

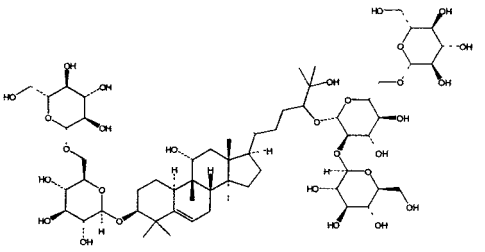
Figure 13: Sample CDXA-09-1623 (UV Chromatogram, Extracted Ion @ 423.2 *m/z*, Extracted Ion @ 439.2 *m/z*, Mass spectra of EIC peaks 1-4)



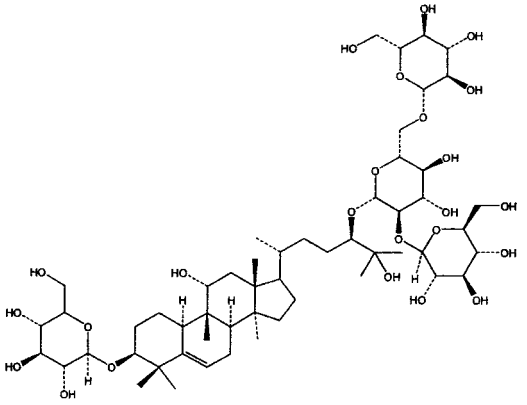
- ***STRUCTURE(S)***



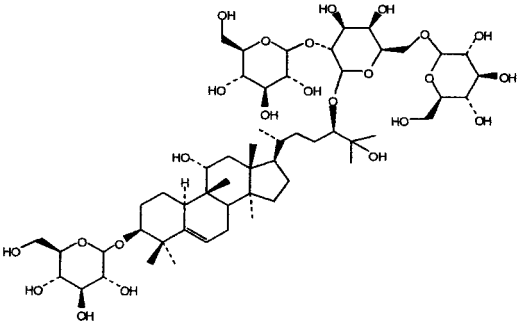
11-Oxo-mogroside V



Mogroside V



Siamenoside I



Grosmomoside I

- ***REFERENCES***

ChromaDex Analytics Laboratory Notebook 161, page 33, 35-36
ChromaDex SOP "Routine Laboratory Calculations"
Analytical Method: CDXA-AM-288-00 "Terpene Glycosides in Luo Han Guo by LC/MS."

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Analytical Results Sheet

Customer:	BioVittoria Limited	Report Number:	CDXA-ARS-4075-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD27787
Sample Name:	PureLo		
Sample Lot:	G-2009000	Date Received:	16-Apr-09
CDXA Number:	CDXA-09-1623	Purchase Order:	NA
Assay:	Water Content by Loss on Drying	Date of Report:	27-Apr-09
Part Number:	CDA-00100130-ARS	Page:	1 of 1
Method:	99.1-CD-1.0-000184	Test Location:	Boulder, CO

Analyte	Units	Spec.	Result	Reporting Limit
Water Content	%	NA	2.46	NA

Approved: Kimberly Eastman

Digitally signed by Kimberly Eastman
DN: cn=Kimberly Eastman, o=ChromaDex, ou=CDXA, email=KimE@chromadex.com, c=US
Reason: I am approving this document
Date: 2009.04.27 14:55:29 -06'00'

Signed original on file at CDXA

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Analytical Test Report

Customer:	BioVittoria Limited	Report Number:	CDXA-ATR-1385-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD27787
Purchase Order:	NA	Date Received:	16-Apr-09
Date of Report:	27-Apr-09	Test Location:	Boulder, CO

Assay: Fatty Acids (Total) including EPA/DHA by GC

Part Number: CDA-00100222-ATR

Prepared By: (b) (6) _____ _____
Analytical Chemist — Date

Reviewed By: (b) (6) _____ _____
Quality Assurance — Date

Approved By: Kimberly Eastman _____ _____
Digitally signed by Kimberly Eastman
DN: cn=Kimberly Eastman, o=ChromaDex, ou=CDXA, email=KimE@chromadex.com, c=US
Reason: I am approving this document.
Date: 2009.04.27 14:50:31 -06'00'
Group Leader, Analytical Services — Date

Signed original on file at CDXA

SUMMARY

- ***SAMPLE(S)***

	Lot #	CDXA #
PureLo	G-2009000	CDXA-09-1623

- **RESULTS**

Table 1 – PureLo CDXA-09-1623

Analyte	Units	Spec.	Result	Reporting Limit
Lauric Acid (12:0)	%	-	*BRL	0.002
Pentadecanoic Acid (15:0)	%	-	*BRL	0.0006
Palmitic Acid (16:0)	%	-	0.0053	-
Stearic Acid (18:0)	%	-	*BRL	0.002
Oleic Acid (18:1n9)	%	-	0.0050	-
Linoleic Acid (18:2n6)	%	-	*BRL	0.0009
Lignoceric Acid (24:0)	%	-	*BRL	0.0009
Total Free Fatty Acids	**%**	-	**0.010**	-

*BRL –Below Reporting Limit

ANALYTICAL METHOD

- ***STANDARD(S)*** *All standards supplied by ChromaDex, unless otherwise specified.*

  Nu-Chek Fatty Acid Reference Standard
  Nu-Chek Methyl Tricosanoate Standard

- ***LABORATORY SUPPLIES***

  HP 5890 GC Series II plus with FID
  Rtx™2330 Column, 105 m x 0.25 mm x 0.20 µm
  Analytical Balance
  Heating Block
  Vortex
  Centrifuge
  Assorted and Volumetric glassware
  Syringes and Syringe Filters
  HPLC/GC glass vials and caps

- ***SOLVENTS AND REAGENTS***

  Hydrochloric Acid (HCl)
  Acetyl Chloride
  Methanol (MeOH)
  Ethyl Ether
  Petroleum Ether
  Heptane
  Sodium Chloride (NaCl)
  Sodium Sulfate (Na_2SO_4)
  Milli-Q Water

- ***SOLUTION PREPARATION***

  5N Hydrochloric Acid (HCL)

  5N HCl was prepared by transferring 417 mL of HCl into a 1000 mL volumetric flask. 400 mL of Milli-Q water was added and solution diluted to volume and transferred to a glass container.

  Anhydrous HCl in Methanol

  In a cool bath 100 mL of acetyl chloride was transferred through a taper addition funnel into a 1000 mL tapered neck flask containing 500 mL methanol. The solution was diluted to volume in a 1000 mL cylinder and transferred to an amber glass container.

Ether mix (1:1)
1000 ml petroleum ether and 1000 ml ethyl ether was transferred into a glass container and mixed well.

NaCl Saturated solution
NaCl was dissolved in Milli-Q water to saturation.

- ***STANDARD PREPARATION***

Internal Standard Solution (ISTD)
2.5 mg of methyl tricosanoate was weighed into a 200 mL volumetric flask, diluted to volume with heptane and mixed well.

Stock Standard Solution
The stock standard solution was prepared by weighing Approximately 370 mg of Nu-Chek Prep standard in 13 mL internal standard solution (ISTD) and used as Level 1 Standard.

Mixed Stock Standard Solution

The fatty acids working standards were prepared by taking 5, 2, 1 and 0.5 ml of the stock standard each into a 10 ml volumetric flask diluted to volume with (ISTD) and mixed well.

.

- ***SAMPLE PREPARATION***

Customer Sample(s) – CDXA-09-1623
Samples were prepared in duplicate by weighing approximately 2.0 g of material into a 50 mL centrifuge tube prepared with internal standard. 10 mL of 5N HCl was added and heated in a heat block at 100°C for 60 minutes. The samples were cooled to room temperature and 10 mL of NaCl saturated solution and 10 mL of the Ether Mix was added. Samples were vortexed for 30 seconds and centrifuged for 2 minutes. The organic layer was transferred into a 50 ml beaker and the extraction repeated two more times, for a total of three extractions. Anhydrous Sodium sulfate was added to each beaker to dry any water dissolved in the ethers. The content of the beaker was transferred into a new 50 mL centrifuge tube and was evaporated with a stream of Nitrogen until it was completely dry. Once dried the sample was derivatized by adding 18 mL of anhydrous HCl in MeOH and placed in a heat block at 100°C for 60 minutes. Samples were cooled to room temperature and 5 mL of heptane and 10 ml saturated solution of NaCl and 10 mL Milli-Q water was added. Samples were diluted 1:5 in GC vials for analysis.

- ***INSTRUMENT PARAMETERS***

Instrument	HP 5890 GC Series II plus
Detection	FID
Column	Rtx-2330 : 105m x 0.25mm x 0.20um
Inlet Temperature	255 °C
Carrier Gas	Helium
Flow Rate	1.0 mL/min
Injection Volume	1.0 µL
Injection Needle Wash	Heptane
Initial Temperature	120 °C
Initial Time	3 min
Injector Temperature	255 °C
Detector Temperature	260 °C
Run Time	50.5 min

Oven Temperature Program

Level	Rate (°C/min)	Final Temp (°C)	Final Time (min)
Initial	N/A	120	3.0
1	4.0	210	5.0
2	2.0	250	0.0

DATA

- ***FIGURES***

Figure 1: Fatty Acids Mixed Standard

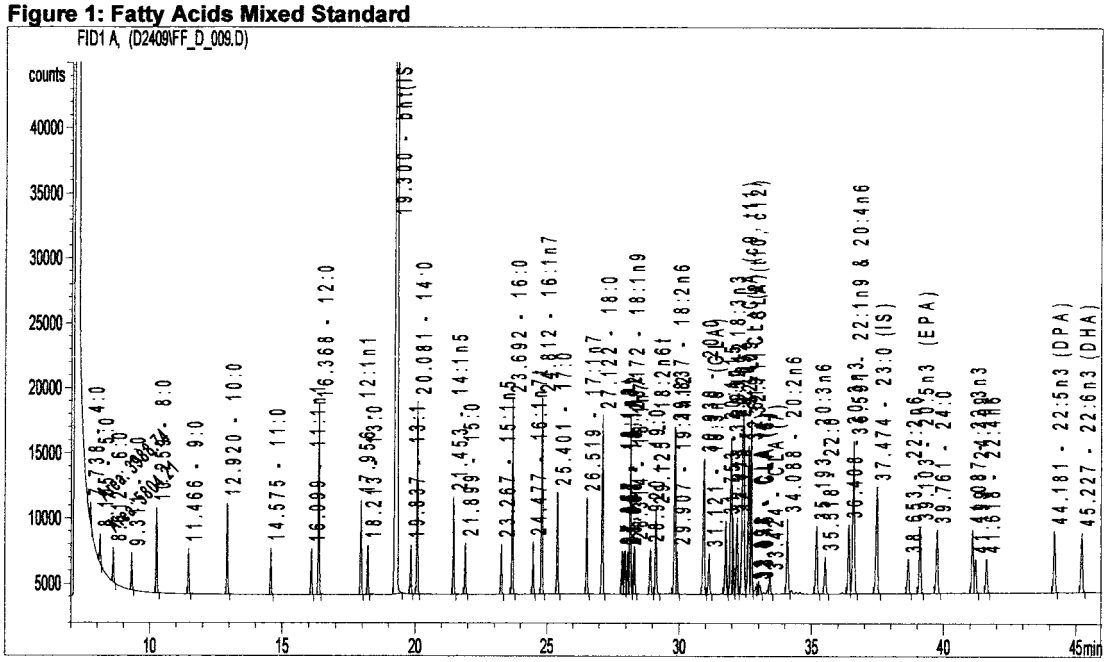


Figure 2: PureLo CDXA-09-1623

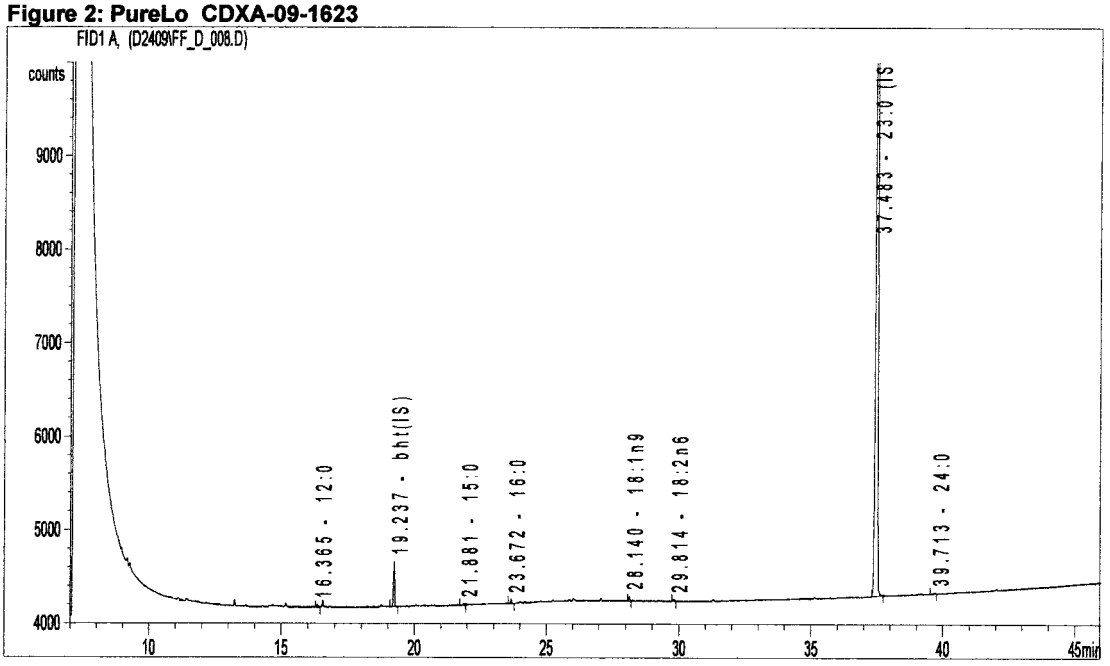
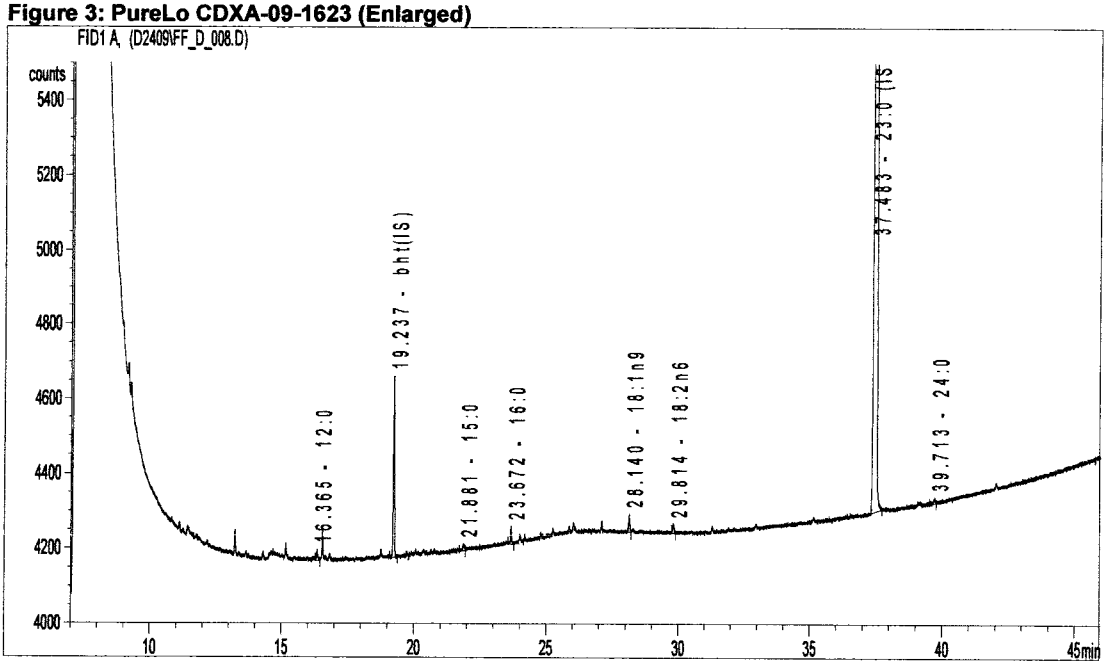
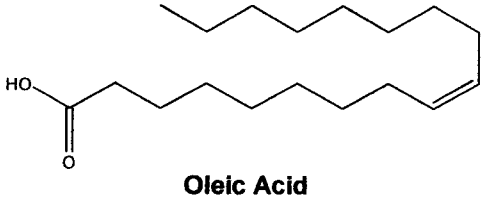


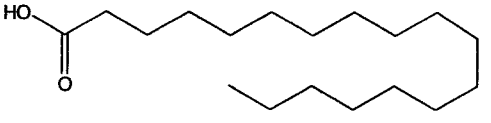
Figure 3: PureLo CDXA-09-1623 (Enlarged)



- **STRUCTURE(S)**



Oleic Acid
18:1n9



Stearic Acid
18:0

- ***REFERENCES***

ChromaDex Analytics Laboratory Notebook 123, page 112-113
ChromaDex Analytics Laboratory Notebook 116, page 55,134,160,163
ChromaDex SOP "Routine Laboratory Calculations"
Analytical Method: 99.1-CD-2.0-000082 "Fatty Acid Analysis by GC or GC-MS"

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Analytical Test Report

Customer:	BioVittoria Limited	Report Number:	CDXA-ATR-1386-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD27841
Purchase Order:	27841	Date Received:	17-Apr-09
Date of Report:	27-Apr-09	Test Location:	Boulder, CO

Assay: Total Protein by BCA (Bicinchoninic acid) Method

Part Number: CDA-00100312-ATR

Prepared By: (b) (6) _____ Analytical Chemist _____ Date

Reviewed By: (b) _____ Quality Assurance _____ Date

Sylesh Venkataraman, Ph.D
Digitally signed by Sylesh Venkataraman, Ph.D
DN: cn=Sylesh Venkataraman, Ph.D, o=ChromaDex Analytics, ou, email=sylesh@chromadex.com, c=US
Date: 2009.04.27 14.11 03 -06'00'

Approved By: **Sylesh Venkataraman** _____ Manager, Chemistry Developments _____ Date

Signed original on file at CDXA

SUMMARY

- ***SAMPLE(S)***

	Lot #	CDXA #
PureLo Part 2	G-2009000	CDXA-09-1623

- ***RESULTS***

Table 1 - CDXA-09-1623

Analyte	Units	Spec.	Result	Reporting Limit
Total Protein	%	-	21.1	-

ANALYTICAL METHOD

- ***STANDARD(S)*** *All standards supplied by ChromaDex, unless otherwise specified.*

	Lot #
Bovine Serum Albumin at 2 mg/mL in 0.9% Saline and 0.05% Sodium Azide (Thermo Scientific)	IL118297

- ***LABORATORY SUPPLIES***

  Analytical Balance; CDXA-CO-204
  Drying Oven
  Assorted and Volumetric glassware and plastic ware
  Syringes and Syringe Filters; Lot# 21678627
  Tecan Microplate reader
  Fisherbrand flat bottom 96 well plate; Lot# 625387

- ***SOLVENTS AND REAGENTS***

  BCA Reagent A™, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide; Lot# HF104262
  BCA Reagent B™, containing 4% cupric sulfate; Lot# HE104697
  HyQ PBS/Modified (1X); Lot# ASH30026
  Working Reagent - 25 mL of BCA Reagent A™ with 0.5 mL of BCA Reagent B™

- ***STANDARD PREPARATION***

The BSA diluted standards were prepared by diluting the 2 mg/mL BSA stock standard with PBS see **Table 2** below.

Table 2. Albumin (BSA) Standard Preparation

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0 µL	300 µL of Stock	2,000 µg/ml
B	125 µL	375 µL of Stock	1,500 µg/ml
C	325 µL	325 µL of Stock	1,000 µg/ml
D	175 µL	175 µL of vial B dilution	750 µg/ml
E	325 µL	325 µL of vial C dilution	500 µg/ml
F	325 µL	325 µL of vial E dilution	250 µg/ml
G	325 µL	325 µL of vial F dilution	125 µg/ml
H	400 µL	100 µL of vial G dilution	25 µg/ml
I	400 µL	0 µL	0 µg/ml = Blank

- ***SAMPLE PREPARATION***

Sample was prepared by weighing 247.30 mg of material into 50 mL sterile culture tubes, adding 10 mL of phosphate buffer solution (PBS). The sample was then vortexed and shaken to extract all proteins. An aliquot was then filtered using a 0.8 µm Versapor syringe filter and syringe. A [1:10] dilution was made by adding 1 mL of the filtrate to 9 mL of PBS.

- ***ANALYTICAL PROCEDURE***

0.025 mL of each standard and sample triplicate was pippetted into the appropriate microwell in a 96-microwell plate. 0.2 mL of the working reagent was added to each well. The plate was then tapped against palm for 30 seconds then incubated at 35 °C for 30 minutes. The plate was then cooled to room temperature and transferred the spectrophotometer for analysis.

Spectrophotometer	Tecan Plate Reader
UV Detection	540 nm

DATA

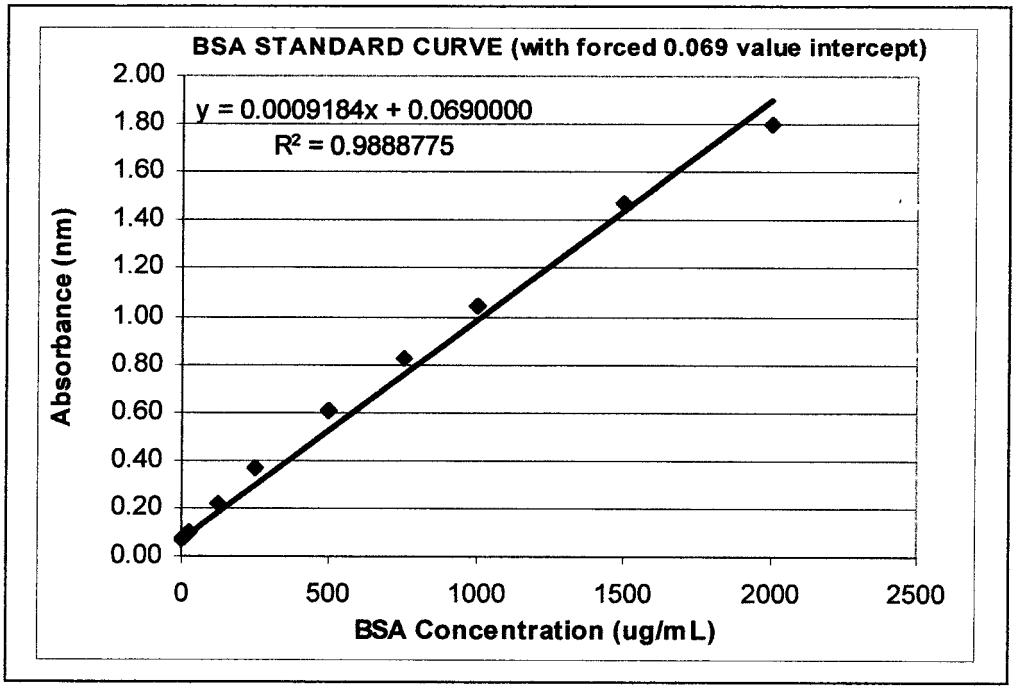
- ***FIGURES***

The absorbencies are described below **(Table 3)** along with the corresponding calibration curve **(Figure 1)**.

Table 3. Absorbancies and Calculated Protein Concentration

Sample	Abs 540nm	Protein (mg/mL)
1	0.538	0.5107
2	0.546	0.5194
3	0.563	0.5379
Average	**0.549**	**0.5226**

Figure 1. BSA Standard Curve



- ***REFERENCES***

ChromaDex Analytics Laboratory Notebook 160, page 6-8
ChromaDex SOP "Routine Laboratory Calculations"
ChromaDex SOP "Protein Quantitation"

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Analytical Results Sheet

Customer:	BioVittoria Limited	Report Number:	CDXA-ARS-4078-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD27787
Sample Name:	PureLo		
Sample Lot:	G-2009000	Date Received:	16-Apr-09
CDXA Number:	CDXA-09-1623	Purchase Order:	NA
Assay:	Residue on Ignition – Ash	Date of Report:	24-Apr-09
Part Number:	CDA-00100066-ARS	Page:	1 of 1
Method:	USP281	Test Location:	Sub15

Analyte	Units	Spec.	Result	Reporting Limit
Residue on Ignition	%	NA	1.57	NA

Approved: Kimberly Eastman

Digitally signed by Kimberly Eastman
DN: cn=Kimberly Eastman, o=ChromaDex, ou=CDXA, email=KimE@chromadex.com, c=US
Reason: I am approving this document.
Date: 2009.04.24 17:09:32 -06'00'

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Analytical Results Sheet

Customer:	BioVittoria Limited	Report Number:	CDXA-ARS-4464-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD28606
Sample Name:	PureLo		
Sample Lot:	G-2009000	Date Received:	02-June-09
CDXA Number:	CDXA-09-1623	Purchase Order:	N/A
Assay:	Total Dietary Fiber	Date of Report:	16-Jun-09
Part Number:	CDA-00100306-ARS	Page:	1 of 2
Method:	AOAC 991.43	Test Location:	Sub20

Analyte	Units	Spec.	Result	Reporting Limit
Total Dietary Fiber	%	N/A	ND	0.1

*N/A = Not Applicable

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ND – Not Detected
BRL – Below reporting limit (compound detected below RL)

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Analytical Results Sheet

Customer:	BioVittoria Limited	Report Number:	CDXA-ARS-4464-00
Address (City, State):	Hamilton, New Zealand	Project Number:	ORD28606
Sample Name:	PureLo		
Sample Lot:	G-2009000	Date Received:	02-June-09
CDXA Number:	CDXA-09-1623	Purchase Order:	N/A
Assay:	Sugars Analysis by GC	Date of Report:	16-Jun-09
Part Number:	CDA-00100274-ARS	Page:	2 of 2
Method:	AOAC 977.20	Test Location:	Sub20

Analyte	Units	Spec.	Result	Reporting Limit
Fructose	%	N/A	ND	0.1
Glucose	%	N/A	ND	0.1
Sucrose	%	N/A	2.78	N/A
Maltose	%	N/A	ND	0.1
Lactose	%	N/A	ND	0.1
Total Sugars	%	N/A	2.78	N/A

*N/A = Not Applicable

Approved: Kimberly Eastman

Digitally signed by Kimberly Eastman
DN: cn=Kimberly Eastman, o=ChromaDex, ou=CDXA, email=KimE@chromadex.com, c=US
Reason: I am approving this document.
Date: 2009.06 16 16.46 47 -06'00'

Signed original on file at CDXA

ND – Not Detected
BRL – Below reporting limit (compound detected below RL)

APPENDIX III

CONCLUSION AND SIGNATURES OF THE GRAS EXPERT PANEL

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF LUO HAN FRUIT CONCENTRATE AS A FLAVOR MODIFIER AND SWEETENER

Prepared for:

BioVittoria Limited
Hamilton City, New Zealand

May 2009

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF LUO HAN FRUIT CONCENTRATE AS A FLAVOR MODIFIER AND SWEETENER

We, the members of the expert panel, have individually and collectively critically evaluated the publicly available information on PureLo® Luo Han fruit concentrate summarized in a monograph prepared by JHEIMBACH LLC, as well as other material deemed appropriate or necessary. Our evaluation included review of starting materials and methods of manufacture of PureLo®, the intake of Luo Han fruit concentrate expected to result from its intended use, both published and unpublished toxicity studies, and the history of consumption of Luo Han fruit decoctions and extracts in the U.S. and elsewhere. Our summary and conclusion resulting from this critical evaluation are presented below.

Summary

- The substance that is the subject of this generally recognized as safe (GRAS) determination is PureLo® Luo Han fruit concentrate, a food produced by decoction and concentration of the Luo Han fruit.

- Production of PureLo® fruit concentrate employs methods similar to those used to prepare traditional decoctions and common to the production of juice concentrates of other fruits. The food is not chemically altered other than by the removal of pectin and sugars using an adsorbent resin, and thus retains the characteristic composition of Luo Han fruit. Appropriate specifications have been established to ensure a food-grade and wholesome product

- The sweetness of Luo Han fruit concentrate is estimated to be approximately 95 times that of sucrose; it is intended to be used alone, as a component of sweetener blends, or added to foods as a sweetener and flavor modifier. The estimated daily intake of Luo Han fruit concentrate, if it were to replace all other intense sweeteners, would be about 6.8 mg/kg bw, or about 475 mg/day for a 70-kg individual.

- The safety of PureLo® Luo Han fruit concentrate has been established by published and unpublished research, including *in vitro* assays for cytotoxicity and anti-inflammatory effects; studies of acute, subacute, and subchronic oral toxicity; and genotoxicity assays. It is further supported by a number of studies in humans, including tests of its effects on blood glucose and liver enzymes.

- Luo Han fruit, in both fresh and dried forms as well as decoctions of the fruit, has long been consumed in China for its sweetening effect; it has also been used as a folk remedy for a variety of benefits that have not been scientifically investigated. It has been imported into the United States for at least a century.

- A number of patents have been granted for various means of extracting and concentrating the juice of the Luo Han fruit, mostly based on use of a chemical solvent, and a large variety of such extracts are available in the U.S. and elsewhere for use as sweeteners and flavor modifiers. Luo Han fruit extracts are also sold in the U.S. as dietary supplements with suggested doses similar to the maximum potential intake estimated to result from the use of Luo Han fruit concentrate as a sweetener.

- The estimated intake of mogrosides and other components of Luo Han fruit from the intended use of PureLo® Luo Han fruit concentrate is of the same order of magnitude as results from traditional uses of dried Luo Han fruit and decoctions of the fruit.

Conclusion

We, the undersigned expert panel members, have individually and collectively critically evaluated the materials summarized above and conclude that:

> Ingestion of PureLo® Luo Han fruit concentrate from the proposed uses results in a level of intake that remains within safe limits established by published and unpublished *in vitro*, animal, and human studies and corroborated by the long history of safe consumption of Luo Han fruit, decoctions, and extracts. PureLo® Luo Han fruit concentrate has been sufficiently characterized to ensure that it is a safe and wholesome food. Therefore, PureLo® Luo Han fruit concentrate meeting the specifications described in the GRAS monograph is safe for use as a sweetener and flavor modifier.

It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, PureLo® Luo Han fruit concentrate is safe, and is GRAS based on scientific procedures, when used as a sweetener and flavor modifier.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus, Toxicology and Pharmacology
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature (b) (6) Date: 28 May 2009

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, Virginia

Signature: _____ Date: _____

John A. Thomas, Ph.D.
Professor Emeritus, Toxicology and Pharmacology
University of Texas Health Science Center
Fishers, Indiana

Signature: _____ Date: _____

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Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature: _____ Date: _____

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, V[illegible]

Signature: _____ Date: May 28 2019

John A. Thomas, Ph.D.
Professor Emeritus, Toxicology and Pharmacology
University of Texas Health Science Center
Fishers, Indiana

Signature: _____ Date: ___ _____

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Arlington, Virginia

Signature: _____ Date: _____

John A. Thomas, Ph.D.
Professor Emeritus, Toxicology and Pharmacology
University of Texas Health Science Center
Fishers, Indiana

Signature: _____ Date: 5/29/09

SUBMISSION END